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A Major Cell Envelope Protein Of Escherichia Coli K12: Purification And Properties

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A MAJOR CELL ENVELOPE PROTEIN OF *ESCHERICHIA COLI* K12: PURIFICATION AND PROPERTIES

by

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ABSTRACT

This thesis reports on the isolation and characterization of a major cell envelope protein from Escherichia coli K12, the so-called "succinate eluted protein" (SEP). SEP was released by EDTA-sucrose and osmotic shock treatments, or during the formation of spheroplasts. The protein was purified on columns of aspartate-Sepharose (A-S), an affinity matrix previously used for the isolation of the dicarboxylate transport components. SEP was the major protein eluted with succinate when the A-S columns were equilibrated in low ionic strength buffer (10 mM phosphate, 5 mM EDTA). SEP was purified to apparent homogeneity as judged by SDS-PAGE, IEF, and HPLC/gel filtration. SEP was able to stimulate succinate transport in whole cells when added externally. To investigate the role of SEP in succinate transport, the binding of [^{35}S]-SEP to whole cells was examined. SEP was able to bind to whole cells with a strong affinity (K_D = 30 nM). The binding of SEP was dependent on the presence of porin and normal K12 LPS. Mutants that lacked porin, or had a very short LPS core were unable to bind SEP or transport succinate. Binding of SEP to isolated porin immobilized on Sepharose was also demonstrated. This binding was very specific in the presence of low concentrations of SDS and was fairly resistant to disruption by NaCl. Binding of SEP to whole cells or porin-Sepharose was not affected by dicarboxylic acids. Studies undertaken to optimize the purification of SEP indicated that increased succinate transport activity and increased levels of SEP occurred when the cells were grown in minimal media. In minimal media the 56K SEP comprises 20-25% of the

proteins released by osmotic shock. These studies also indicated that the interaction of SEP with A-S was not that strong as the protein could be eluted with 30 mM phosphate, differentiating it from the previously characterized 16K periplasmic dicarboxylic acid binding protein (DBP) which was retained by A-S equilibrated in 50 mM phosphate and 50 mM arsenate. Elution with 30 mM phosphate was used to isolate increased amounts of SEP free of other proteins or succinate. No succinate binding to SEP could be detected using a number of rapid binding assays. It was speculated that the mechanism of action of SEP in stimulating the transport of negatively charged succinate may involve the blocking of charged groups close to the opening of the porin channel.

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NOMENCLATURE

(1) Abbreviations

A	Absorbance
A-S	Aspartate-Sepharose
ATP'ase	Adenosine 5°-triphosphatase
BCIP	5-Bromo-4-chloro-3-indoyl phosphate
BSA	Bovine serum albumin
cAMP	Cyclic 3'-5'-adenosine monophosphate
CPM	Counts per minute
DBP	Dicarboxylate binding protein (periplasmic)
DKF	DK-factor
DNA	Deoxyribonucleic acid
DPM	Decays per minute
EDTA	Ethylenediaminetetraacetic acid
g	Gravitational constant
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane-sulfonic acid
HPLC	High performance liquid chromatography
IEF	Isoelectric focusing
IgG	Immunoglobulin G
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
K_D	Dissociation constant
K_i	Inhibition constant
K_m	Michaelis-Menten constant
LPS	Lipopolysaccharide
MBP	Maltose binding protein (periplasmic)
MDO	Membrane-derived oligosaccharides

NOMENCLATURE (Continued)

ME	2-Mercaptoethanol
NBT	Nitro blue tetrazolium
NEM	N-ethylmaleimide
OBP	Oligopeptide binding protein
OD	Optical density (Absorbance)
Omp	Outer membrane protein
ONPG	o-Nitrophenyl-β-D-galactopyranoside
PAGE	Polyacrylamide gel electrophoresis
pI	Isoelectric pH
PMSF	Phenylmethylsulfonyl fluoride
PTS	Phosphoenolpyruvate dependent carbohydrate phosphotransferase system
P.S.I.	Pounds per square inch
RNA	Ribonucleic acid
SBP	Succinate binding protein (cytoplasmic membrane)
SDS	Sodium dodecyl sulfate
SEP	Succinate eluted protein
TBS	Tris buffered saline
TBST	TBS containing 0.05% Tween-20
TCA	Trichloroacetic acid
Tris	Tris(hydroxyl methyl)aminomethane
V_{max}	Maximum rate of Michaelis-Menten

(II) Commonly Used Terms

Cell Envelope	In *E. coli* consists of (from the outside); the outer membrane with the tightly associated peptidoglycan layer; the periplasmic space (an intermembranous aqueous (?) compartment); and the cytoplasmic (inner) membrane.
Cell Surface Components	Any component exposed to the environment on the surface of a cell. In *E. coli* these would be exposed integral outer membrane components (e.g. porins or LPS) or peripheral proteins on the outside of the outer membrane. Cell surface components can be distinguished by their accessibility to agents (i.e. proteins and other compounds) which cannot cross the outer membrane.
K	In terms of molecular weight, refers to ,000's. (e.g. the 56,000 molecular weight species is referred to as 56K).
Microfuge	A small fixed speed microcentrifuge (e.g. Eppendorf Model 5412 or Fisher Model 235B) capable of generating about 11,000 x *g*.
Microfuge Tubes	Polypropylene 1.5 ml snap-cap tubes designed for use in the microcentrifuge.
Pass through	In terms of column chromatography, that part of the material loaded on to the column which is not retained by the column.
Spheroplasting	The action of forming *E. coli* spheroplasts.
Transport	The process by which a compound crosses a membrane into a cell.
Uptake	The measured influx of a substrate into a cell; may involve a combination of transport and metabolism.

CHAPTER 1

AN OVERVIEW OF THE CELL ENVELOPE AND TRANSPORT MECHANISMS IN *ESCHERICHIA COLI*

1.1 INTRODUCTION

"In all sanguinous animals membranes are found. And membranes resemble a thin close-textured skin, but its qualities are different, as it admits neither to cleavage or extension..."
Aristotle (384-322 B.C., *History of Animals*, III.13)

"Permeability in so far as it concerns physiology is that property which permits water and substances dissolved in water to pass through the limiting surfaces of a cell or tissue, as through an inert membrane separating two different aqueous solutions, one from the other. The relative speeds of diffusion and osmosis permit deductions as to the composition as well as physical properties of the membranes which play a role in the passage."
Rudolf Höber, "Permeability", Annual Review of Biochemistry, Vol. 1, page 1, (1932).

From the preceeding quotations it is obvious that the study of membranes and their permeability properties is a subject of long standing interest. And justly so, for it was a membrane that allowed the 'primordial substance' to be surrounded and isolated from its environment, so forming the first cell and allowing life, as we know it, to evolve. As life became more complex so, necessarily, did the membrane until today we are faced with membranes in a myriad of forms, with a myriad of functions; enough to provide membrane biochemists with many lifetimes of experiments, wonderment, and problems.

This thesis deals with one of these forms, the cell envelope of *Escherichia coli*, and one of these functions, the transport of dicarboxylic acids through it.

1.2 THE CELL ENVELOPE OF ESCHERICHIA COLI

To maintain life, a membrane must be both protective and allow passage of those compounds necessary to sustain life, at such a rate as to allow growth and division, hence the evolution of specific transport systems.

Prokaryotic cells, and in this case the Gram-negative bacterium Escherichia coli, have devised elaborate cell envelope structures to allow them to maintain a free living existence under a variety of environmental conditions. Escherichia coli is an enteric bacteria, and survives in the gut in an environment containing proteases and hydrophobic compounds such as bile salts. The cell envelope consists of a cytoplasmic membrane (analogous to the plasma membrane of eukaryotes), the periplasmic space, and an outer membrane with a tightly associated underlying peptidoglycan layer. While nothing is known about cell surface peripheral proteins in E. coli, other species of bacteria may have external cell surface arrays, lending complexity to the whole structure. A schematic representation of the Escherichia coli cell envelope is presented in Fig. 1.1.

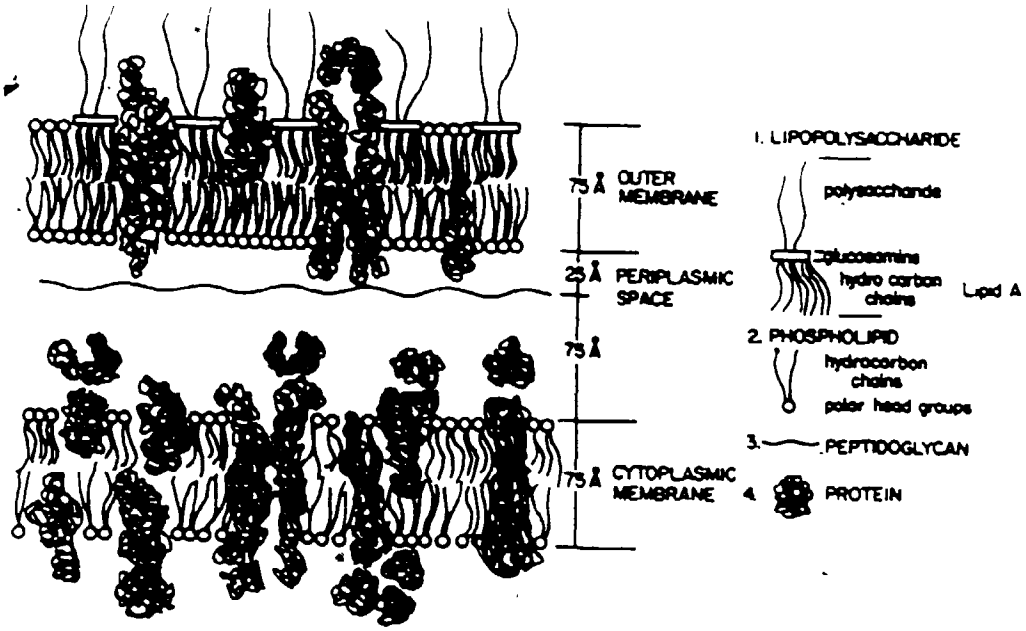
1.2.1 The Outer Membrane

The first permeability barrier encountered by an incoming metabolite is the outer membrane. The outer membrane (in conjunction with the tightly associated underlying peptidoglycan layer) has a number of functions including protection against lysis in hypo-osmotic media; resistance to certain chemicals, enzymes, antibiotics and bile salts; and the compartmentalization of hydrolytic enzymes in the periplasmic space, preventing their loss into the media (as is the case in Gram-positive bacteria) (1).

FIGURE 1.1

THE CELL ENVELOPE OF ESCHERICHIA COLI

Schematic diagram showing the macromolecular constituents (proteins, phospholipids, lipopolysaccharide, and peptidoglycan) of the outer membrane, periplasmic space, and cytoplasmic membrane. Reproduced with permission from T. Lo (51).


75 Å OUTER MEMBRANE
25 Å PERIPLASMIC SPACE
75 Å
75 Å CYTOPLASMIC MEMBRANE
1. LIPOPOLYSACCHARIDE
polysaccharide
glucosamine
hydro carbon chains
Lipid A
2. PHOSPHOLIPID
hydrocarbon chains
polar head groups
3. PEPTIDOGLYCAN
4. PROTEIN

The outer membrane is a bilayer structure, containing a few species of integral membrane proteins in high copy number. The outer leaflet of the bilayer is composed largely of lipopolysaccharide while the inner leaflet contains phospholipids (mainly phosphatidylethanolamine) (2). The outer membrane is associated with the underlying peptidoglycan layer, and together these two structures give the cell its shape and rigidity. The principal proteins of the outer membrane include the porins, which form nonspecific permeability channels through the membrane, as well as Braun's lipoprotein and outer membrane protein A (OmpA). These latter two proteins have a structural role, and in the case of OmpA, a role in conjugation and as a phage receptor (2).

In E. coli the major porins are OmpF and OmpC, proteins with molecular weight around 37,000 and with a large amount of β-structure (3). Their expression is controlled by the ompB locus and depends on the composition and osmotic strength of the environment (3). OmpF is produced in media of low osmotic strength and OmpC in media of high osmolarity. These porins exhibit some selectivity in that OmpF was shown to transport cations at a faster rate than anions, due to the presence of fixed negative charges in the pores (1).

The porins form ordered structures with three identical polypeptides as the basic minimum functional unit (3). These trimers interact with lipopolysaccharide and lipoprotein. Their transmembrane nature is evidenced in that they serve as both phage receptors and are tightly associated with the underlying peptidoglycan layer.

Some species of porin belong to specific transport systems. These include phosphoporin, maltoporin, and phage T6 receptor. Phosphoporin, the phoE gene product, contains a phosphate binding site, and is induced

under conditions of phosphate limitation to facilitate phosphate transport. Maltoporin, a product of the lamB gene, is part of the maltose transport system. It has a binding site for maltodextrins and its production is induced in the presence of maltose. The phage T6 receptor is part of the nucleoside transport system. All of these "specific" porins also allow the nonspecific diffusion of other small molecules.

Lipopolysaccharide (LPS) is the other major component of the outer membrane, covering 45% of the membrane surface. It is exclusively located in the outer leaflet of the bilayer. LPS consists of polymers of complex polysaccharide chains forming the O-specific polysaccharide and the core oligosaccharide to which is linked a unique glucosamine containing lipid, lipid A (4). The lipid A portion substitutes for the phospholipids in the outer leaflet of the bilayer with the polysaccharide chains extending outwards into the external environment. E. coli K12, which is used in our study, lacks the O-specific polysaccharide (4) and contains only the core oligosaccharide linked to lipid A. LPS plays a role as a phage receptor and as a barrier against hydrophobic compounds (e.g. bile salts) (1,2). The association between the negatively charged LPS and negatively charged porins is maintained by divalent cations. Chelating agents and polycations disrupt the integrity of the outer membrane (5).

1.2.2 The Periplasmic Space

After crossing the outer membrane, a compound finds itself in the periplasmic space. The periplasm may contain 20-40% of the total cell volume (6). This compartment contains proteins of three main classes (7); hydrolytic enzymes, such as nucleases, proteases, and peptidases

which serve to degrade incoming compounds; proteins such as β-lactamases, which serve as protective agents against penicillins and toxic substances; and binding proteins for a large number of transport systems. The contents of the periplasmic space can be released by the osmotic shock procedure of Neu and Heppel (8) which involves rapidly shifting the cells from a hyperosmotic medium to a hypo-osmotic medium after disrupting the outer membrane with EDTA in Tris-HCl. Transport activity of binding protein-dependent transport systems is sensitive to osmotic shock, and binding proteins can be isolated from the osmotic shock fluid (7,8).

One of the major components of the periplasmic space is the peptidoglycan network, which is comprised of repeating units of N-acetyl glucosamine and N-acetyl muramic acid linked by β(1->4) glycosidic bonds (9). Hen egg white lysozyme specifically cleaves the linkage between these two sugars in the E. coli peptidoglycan.

The periplasm contains a large amount of fixed negative charge on a family of related compounds called the membrane derived oligosaccharides (MDO). These compounds have a structure containing 6 to 12 glucose units linked by β(1->2) and β(1->6) bonds and are variously substituted with phosphoglycerol, phosphoethanolamine, and O-succinyl ester residues (10). The levels of MDO are regulated by the osmotic strength of the environment reaching 5-7% of the cell's dry weight in media of low osmolarity (11). This allows the cytoplasm to be maintained in an isosmotic environment, even though the external environment changes.

The nature of the periplasmic environment is a subject of some controversy. Hobot et al (12) have suggested that the periplasm is not a simple aqueous compartment but rather is filled with a viscous gel.

In their model the peptidoglycan is more hydrated than previously assumed and is in the state of a gel, filling the entire periplasmic space. It was felt that the peptidoglycan is more highly crosslinked close to the outer membrane and less so towards the cytoplasmic membrane at which it forms a gel layer.

Fluorescence photobleaching recovery experiments done by Brass et al (13) with biologically active, fluorescently labelled, maltose binding protein introduced into the periplasmic space, indicated a very low lateral diffusion coefficient. This coefficient was 1,000-fold lower than would be expected for diffusion in an aqueous medium and almost 100-fold lower than for a protein of equivalent size in the cytoplasm. These results are consistent with a periplasm that is very viscous or highly compartmentalized.

1.2.3 The Cytoplasmic Membrane

After traversing the periplasmic space, a substrate must get across the cytoplasmic membrane before it can be metabolized by the cell. The cytoplasmic membrane is a typical phospholipid bilayer, containing a large number of integral and peripheral proteins, including proteins of the electron transport chain, the F_0F_1-ATPase, biosynthetic enzymes, and transport proteins. A hydrophillic substance, reaching the cytoplasmic membrane, requires a specific transporter for its passage into the cytoplasm at an efficient rate.

1.3 BACTERIAL TRANSPORT SYSTEMS

When discussing transport phenomena in E. coli it is worthwhile to provide examples of well studied transport systems. Bacterial transport systems can be divided into periplasmic binding protein independent or

dependent systems. Well characterized binding protein independent systems include the glycerol transport system, the phosphoenolpyruvate : sugar phosphotransferase system (PTS), and the lactose permease. The maltose transport system, is one of the best studied examples of a binding protein dependent system.

1.3.1 Binding Protein Independent Systems

1.3.1.1 The Glycerol Transport System

The uptake of glycerol involves the translocation through the membrane by means of a facilitator (which acts as a pore), and immediate intracellular conversion to glycerol-3-phosphate by glycerol kinase to prevent exit (14). Solute facilitators equilibrate their substrates across the membrane without accumulating them against a concentration gradient. This is an energy-independent process. The glycerol facilitator contains no binding site for glycerol, and its specificity depends on molecular size and charge of the solute. Another example of a simple facilitator is the outer membrane porin.

1.3.1.2 Phosphoenolpyruvate:Sugar Phosphotransferase System

The phosphoenolpyruvate.dependent carbohydrate phosphotransferase system (PTS) is an example of group translocation in which the transport and concomitant phosphorylation of a series of carbohydrates (PTS carbohydrates) results in the intracellular accumulation of carbohydrate phosphates. The carbohydrate phosphate is the first intermediate in the catabolism of the carbohydrate and provides a tight linkage between uptake and subsequent metabolism so that no free carbohydrate is found in the cell (15). One of the transported carbohydrates is glucose. For

transport of glucose, two soluble proteins Enzl and HPr, and two glucose specific membrane bound proteins, $Enzll^{glc}$ and $Enzlll^{glc}$ are involved. Of these components, only $Enzll^{glc}$ possesses a specific binding site for D-glucose; sugar binding sites cannot be detected in the other components.

1.3.1.3 The Lactose Permease System

Lactose transport was one of the earliest studied active transport systems and remains one of the best characterized. This system transports galactosides with α-(melibiose) or β-(lactose) glycosidic linkages (16).

Membrane vesicles (17) are used to study transport in binding protein independent transport systems, such as the lactose permease. It was shown that lactose accumulated by an active transport process; the uptake was driven by the electrochemical gradient of protons ("proton-motive force") across the membrane of energized vesicles (18), and transport of lactose is accompanied by proton symport.

Although purified lactose carrier has been functionally reconstituted in liposomes, additional proteins may be involved in energy coupling (19). Various pleiotropic mutants have been isolated which are defective in proton-motive force coupled active transport systems. The gene products involved, putative energy coupling proteins, have yet to be characterized (16).

The lactose permease is a symmetric carrier with demonstrated substrate binding sites. Entry and exit are mediated by the same protein. This is in contrast to binding protein dependent systems in which different systems may be involved in entry and exit (16).

1.3.2 Binding Protein Dependent Transport Systems

Binding protein dependent transport systems are characterized by their sensitivity to the osmotic shock procedure of Neu and Heppel (8), which releases periplasmic contents. As well, no uptake is seen in spheroplasts or membrane vesicles. In binding protein dependent systems the specificity is determined by the periplasmic binding protein. The energy source for uptake may be a phosphorylated compound (e.g. ATP or acetyl phosphate (20)) or lipoic acid (21). Proton motive force plays only an accessory role at most, and proton symport does not seem to occur. Considerable substrate gradients (up to $1:10^5$) can be produced (16). One of the best studied examples of binding protein dependent transport is the maltose transport system.

1.3.2.1 The Maltose Operon (malB)

The maltose transport system is inducible by maltose; is coded by the malB operon; and consists of 5 genes; malE, malF, malG, malK and lamB (16). LamB codes for the outer membrane λ-phage receptor (maltoporin). Active maltoporin is a trimer. In cells fully induced for maltose transport up to 10^5 molecules/cell of maltoporin are present.

MalE codes for the 38,000 molecular weight periplasmic maltose binding protein (MBP). In the fully induced state, $3\text{-}4 \times 10^4$ molecules are present per cell. This is about 40% of the periplasmic protein, equivalent to a periplasmic MBP concentration of 1 mM (16). MBP recognizes maltose and higher $\alpha(1\rightarrow 4)$ glucose polymers (maltodextrins). The affinity of MBP for all these compounds is around 1 μM.

The malF, malG, and malK gene products encode the cytoplasmic

membrane transport machinery. MalF codes for a 40,000 molecular weight integral cytoplasmic membrane protein, present in 500–1000 copies/cell. The malG product is a cytoplasmic membrane protein with molecular weight of 24,000. No substrate binding activity has been detected with the cytoplasmic membrane components; instead, MalG and MalF are thought to function as recognition components for MBP (22) as outlined below. The malK product is a 40,000 molecular weight protein, localized at the inner cytoplasmic membrane face. This protein does not exhibit maltose binding activity but contains a consensus nucleotide binding sequence and may be involved in energy coupling (23).

1.3.2.2 Role of Maltoporin in Transport

A specific outer membrane protein (maltoporin) is a necessary component of the maltose transport system. In vivo, (at 1 μM maltose) maltose passes through maltoporin 100 times faster than the rate that it goes through OmpF porin. At this concentration, diffusion through maltoporin is rate limiting; mutants with 10% of wild type levels of maltoporin show 10% of wild type uptake (24).

Periplasmic MBP may help facilitate transport across the outer membrane. At least 5 pieces of evidence indicate a functional interaction between MBP and maltoporin. 1) Ultrastructural studies indicate that the majority of MBP is located at or near the outer membrane (25). 2) Solubilized maltoporin binds tightly to MBP immobilized on Sepharose, and 1 M NaCl is required to elute it (26). 3) Maltoporin mutants, with altered MBP-Sepharose binding, have altered transport rates. 4) Mutants unable to grow on maltodextrins have been isolated. MBP in these mutants exhibits normal substrate binding. MBP-

Sepharose prepared from these mutants did not bind wild type maltoporin (27). 5) In vitro reconstitution studies of Neuhaus et al (28) indicated that MBP stabilized the channel forming capacity of maltoporin in black lipid bilayers, although other studies (Benz et al, 29) using different reconstitution techniques showed no influence of MBP on the pores.

1.3.2.3 Role of Maltose Binding Protein in Transport

As well as its putative involvement with maltoporin, MBP is required for transport of maltose across the periplasmic space to the cytoplasmic membrane. The mechanism by which a periplasmic binding protein increases substrate uptake is unclear. Based on the tight binding of MBP with its substrates (around 1 μM) one would expect a slow down of overall substrate diffusion across the periplasmic space, as the substrate becomes trapped by the binding protein. The tight binding also precludes any increase in free substrate, although the total substrate concentration (bound + free) will increase. Thus it is not the free maltose but the maltose–MBP complex which is thought to be recognized by the cytoplasmic membrane components (16). No maltose binding to membranes or membrane bound components has been demonstrated in wild type cells. Strains carrying a deletion in malE were unable to grow on maltose (even at 25 mM). MalE independent revertants were able to grow but the K_m for transport was 1.7 mM (i.e. about 2,000-fold higher than wild type cells) and a concentration ratio of only 1:10 was obtained (30) (about 10,000 fold lower than wild type).

X-ray crystallographic studies by Quiocho's group using various periplasmic binding proteins including MBP indicate that these proteins

consist of 2 globular lobes separated by a cleft that contains the ligand binding site (31). Binding of ligand causes the 2 lobes to close down upon the molecule, expelling the solvating water molecules, forming extensive hydrogen bonds, and reducing the accessible surface contact area by about 98% (32). This "Venus's flytrap" action results in a binding protein of altered conformation and could allow for the specific interaction of the "liganded form", in preference to the "unliganded form", with the membrane bound components, thus initiating the translocation process. Sequestering of ligands should facilitate transport by excluding the bulk water at the interface between binding proteins and their membrane receptors.

The above data indicate the necessity of binding proteins in these systems, serving as the high affinity ligand receptor site for the otherwise low affinity transport components. Attempts to reconstitute these systems in osmotically shocked cells have consistently failed. However, for ribose and phosphate transport, addition of purified binding proteins to spheroplasts has resulted in a restoration of transport activity (33,34) and in the case of glutamine transport, reconstitution has been accomplished in both spheroplasts and membrane vesicles (20,35). In *malE* deletion mutants (that were constitutive for the rest of the *malB* operon), the addition of MBP to the periplasm by means of a low temperature:high calcium treatment restored maltose transport activity (36).

There is however, no direct biochemical evidence for the interaction between binding proteins and the cytoplasmic membrane transport components, although genetic evidence exists (22,37).

A number of proteins from detergent solubilized cytoplasmic membranes bind to columns of immobilized MBP, in the presence of maltose (38). One of the proteins (molecular weight 62,000) was identified as the chemotaxis protein Tar. Two other proteins that bound with molecular weights 38,000 and 24,000 were unidentified at the time (1979). These molecular weights are similar to the products of malF (40,000) and malG (24,000) implicated by genetic means (22).

1.3.3 The C_4 Dicarboxylic Acid Transport System

Transport of dicarboxylic acids into E. coli was first reported by Kay and Kornberg (39), and later intensively studied by Lo and coworkers (40-49).

The various components of the uptake system were isolated by affinity chromatography on "aspartate-Sepharose" (41-44). This matrix consists of aspartate, coupled through its amino group to a long spacer arm on Sepharose. Coupled in this way the aspartate is thought to resemble succinate. Two membrane components SBP1 (dctB gene product) and SBP2 (dctA product) were isolated (41). Binding studies with right side out and inside-out vesicles showed that the binding site for SBP2 (K_m = 4 μM succinate, 7 μM malate) and SBP1 (K_m = 23 μM succinate, 47 μM malate) are located on the outer and inner surface of the cytoplasmic membrane, respectively (42, 43). Approximately 3,000 binding sites were located on the outside of the cytoplasmic membrane of each cell.

The presence of a periplasmic binding protein was determined by the osmotic shock sensitivity of dicarboxylic acid transport and the isolation by affinity chromatography of a binding protein from the osmotic shock fluid (44). This protein, the dicarboxylate binding protein (DBP) also bound lactate weakly. A molecular weight of 16,000

and a K_D for succinate of 40 μM was found. Succinate binding was inhibited by fumarate, malate, and lactate with K_i's of 55 μM, 34 μM, and 1.3 mM respectively (44).

Populations of DBP could be distinguished by labelling with various impermeant reagents followed by selective disruption of the cell envelope layers, and separation on aspartate–Sepharose. These studies indicated that DBP is present at the cell surface (45); in the outer region of the periplasmic space (i.e. between the outer membrane and peptidoglycan); and in the inner region of the periplasmic space (i.e. between the peptidoglycan and cytoplasmic membrane)(46). These populations of DBP could be released by EDTA–sucrose, osmotic shocking, and a post–osmotic shock lysozyme treatment, respectively.

Transport of succinate across the outer membrane of Escherichia coli at low concentrations of substrate, is rate limiting and is dependent on the presence of porins (47). Mutants that lack certain porins in the outer membrane were shown by Lo and Bewick (47), to be markedly ineffective at succinate transport at low succinate concentrations.

Protease treatment of whole cells caused a diminution of succinate transport (45). This was not felt to be due to proteolysis of porin (which is remarkably protease resistant in vivo) (2) but rather to proteolysis of the cell surface DBP. By use of a nonpenetrating substrate analogue, aspartate–dextran, and through reconstitution studies with purified DBP (in which the addition of DBP to whole cells was shown to stimulate succinate uptake), the cell surface DBP was postulated to play an important role in succinate influx (47). A model

was proposed in which the interaction between the cell surface DBP and the nonspecific transport channel formed by porin, would increase the efficiency of the porin channel by conferring upon it, in effect, an substrate binding site in the external media. Together, this "DBP-porin channel complex" was thought to form an efficient transport system for dicarboxylic acids (47)

The dicarboxylic acid transport system, with demonstrated uptake in membrane vesicles (48,49), an apparent dependence on proton motive force (49) and co-transport with protons (50), differs from the "prototypic" osmotic shock sensitive binding protein dependent system. Although this system was osmotic shock sensitive, strong substrate binding to the membrane transport components was demonstrated. The dicarboxylic acid transport system may thus be classified as a binding protein "assisted" system to distinguish it from systems with an absolute binding protein dependence.

1.4 OBJECTIVES

In this thesis I will examine the properties of a major protein isolated from the osmotic shock fluid by aspartate-Sepharose affinity chromatography, and discuss its possible role in facilitating the transport of dicarboxylic acids across the cell envelope. This protein, called the succinate eluted protein (SEP) was the major protein species isolated by elution of aspartate-Sepharose columns with succinate, when the columns were loaded at low ionic strengths. A number of physicochemical methods were utilized to determine both the properties and purity of the isolated protein. These methods included SDS and isoelectric focusing gel electrophoresis, high performance liquid

chromatography (HPLC) gel filtration and peptide mapping, and amino acid analysis.

Characterization of a newly isolated protein by physicochemical means is necessary for a number of reasons. Firstly it provides valuable information on the properties, which may then suggest alternate routes of isolation. This information may also be used in screening mutants, if the protein of interest can be easily discerned amongst other proteins. When the protein of interest does not contain easily assayable biological activity (as is the case with binding proteins and structural proteins) this physicochemical information is particularly important. Second, physicochemical studies are essential for comparative purposes, allowing one to determine if a similar protein has been previously isolated. Third, manipulation of an isolated protein in various *in vitro* situations could cause a change in properties. Reanalysis of the modified protein may provide an indication of the nature of the change. Fourth, careful physicochemical analysis could provide an indication of unusual properties and avenues for further research.

To study the involvement of a protein in a complex cellular process, such as the transport of succinate, requires that one must establish a firm foundation for experimental design. The use of sub-optimal conditions can lead to one "working on the edge of detectability" with any minor procedural changes leading to wide fluctuations in results. The optimization of experimental conditions is of utmost importance in defining "the grey areas" and allowing one to determine if changes in experimental results are real. To this end considerable time was spent in optimizing the conditions of SEP isolation.

Based on the finding that SEP can activate succinate transport when added externally it was thought that the protein might be involved in succinate transport. Conditions of growth (medium, growth phase, and aeration) which provided optimum uptake ability and SEP production were determined. As growth conditions can affect the fragility of membranes, the conditions of SEP isolation were investigated using optimally grown cells.

The determination of the subcellular localization of a protein is particularly important to both determining its role in transport and optimizing its isolation. The assignation of a protein to a cell envelope location requires careful controls. Release of a protein by osmotic shock is often used as adequate reason for this classification without sufficient consideration being given to the question of generalized cell lysis. Beacham (7) noted that this is a risky proposition, especially if the osmotic shocking is into water where cytoplasmic membrane damage and release of cytoplasmic contents can occur. Proteins that are presumably located on the inner cytoplasmic membrane surface (most notably elongation factor Tu (52)) can be released by osmotic shock even when other cytoplasmic membrane components are retained. Spheroplast formation has been reported as a more gentle way to release periplasmic contents, without release of these peripheral proteins. Spheroplast formation involves disrupting the integrity of the outer membrane with Tris and EDTA, and digestion of the peptidoglycan layer with hen egg white lysozyme (53), no harsh hyperosmotic-hypo-osmotic shock is involved. We used spheroplast formation in conjunction with optimized osmotic shock to confirm the cell envelope location of SEP.

The aspartate–Sepharose affinity chromatography running conditions were also investigated in detail. This provides information on the affinity of the protein for the matrix. This gives an indication of the properties of the protein, and also allows one to establish elution conditions for obtaining the maximum amounts of pure protein.

The ability of the purified SEP to bind succinate was examined. Because SEP could stimulate whole cell uptake when added externally, the ability of purified SEP to interact with whole cells was examined. SEP was able to bind with whole cells, and demonstrated a degree of specificity, in that the presence of porin was required. To investigate the porin–SEP interaction, the binding of SEP to porin immobilized on Sepharose was studied.

CHAPTER 2

ISOLATION AND PHYSICOCHEMICAL CHARACTERIZATION OF THE SUCCINATE ELUTED PROTEIN (SEP)

2.1 INTRODUCTION

The dicarboxylate binding protein (DBP) of Escherichia coli K12 has been shown to be an important component of the transport system for dicarboxylic acids (45). The original isolation procedure involved osmotic shocking into cold water and separation of the released proteins on an affinity matrix of "aspartate-Sepharose" (44) in the presence of 50 mM phosphate and 50 mM arsenate. The protein was eluted from the column with 200 mM succinate.

Retention on aspartate-Sepharose (in the presence of 10 mM phosphate) was used by Bewick and Lo (46) to quantitate populations of DBP in various locations within the cell envelope. The amount of protein bound to the column was used as a measure of "releasability" by various treatments. Three populations of protein were detected. One population comprising about 10% of the total, was localized to the cell surface. This population was released by an EDTA-sucrose treatment of the cells, was preferentially labelled with membrane impermeant reagents, and contained bound LPS. A second population of DBP was released by osmotic shocking into water, and was thought to be composed of "free" periplasmic protein. A third population of protein was released by lysozyme treatment of the shocked cells, and was believed to contain DBP bound to, or retained by, the peptidoglycan layer.

We were originally interested in studying DBP, especially its role in the transport of dicarboxylic acids across, and its interaction with,

the outer membrane. To this end we set out to isolate DBP from E. coli according to the methods of Bewick and Lo (46) with a number of modifications.

Initial experiments by Lo and Sanwal had reported a molecular weight for DBP of around 16K (44). No measures to prevent proteolytic degradation had been used. Later work in this laboratory had indicated that a protein, isolated by affinity chromatography, had a higher molecular weight but storage at 4°C resulted in a reduction in molecular weight (T.C.Y. Lo, personal communication) indicative of proteolytic degradation. We undertook to isolate DBP in an undegraded form through the use of the phenylmethylsulfonyl fluoride (PMSF) an inhibitor of periplasmic serine proteases (54), and EDTA, to inhibit divalent cation activated proteases (shown by R.A. Cook of this department to be important proteases of the periplasmic space (55)). As well, the immediate quick freezing of isolated subcellular fractions and purified protein was implemented. The purified protein was then characterized by various physicochemical methods. As will be indicated later, this procedure resulted in the isolation of a major cell envelope protein, the properties of which differ considerably from those previously determined for DBP. Since this is a major protein eluted from the aspartate-Sepharose upon the addition of succinate, it is therefore referred to as the "succinate eluted protein" or SEP.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 Bacterial Strains

Escherichia coli K12 strain CBT43 (45) (sdh, frd, thi) was used in this study. This strain will grow on malate or glycerol but not

succinate or acetate as a carbon source in the presence of thiamine (vitamin B_1). Growth phenotype was routinely checked by replica plating onto minimal medium A plates (56) containing 0.2% of the carbon source. The plates were checked for growth after incubation for 48 hr at 37°C.

2.2.2 Growth Media

Growth media were as described by Miller (56). LB is a rich medium containing (per litre) 10 g Bactotryptone (Difco), 5 g yeast extract (Difco), and 10 g NaCl. The pH was adjusted to 7.2 with KOH. M9 is a sulfate-free minimal salts medium containing (per litre) 6 g dibasic sodium phosphate, 3 g monobasic potassium phosphate, 2 g ammonium chloride, and is supplemented after autoclaving with 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.001% vitamin B_1, 10 ml of LB per litre, 0.2% glycerol as carbon source, and 0.2% succinate to induce the dicarboxylate transport system.

2.2.3 Preparation of Aspartate-Sepharose

The preparation of aspartate-coupled Sepharose-4B was as outlined by Lo (57). The Sepharose was stored in 10 mM Tris-HCl (pH 8.0) and 5 mM EDTA at 4°C.

2.2.4 Preparation of [^{35}S]-Labelled Osmotic Shock

Preliminary experiments had indicated that we were able to metabolically label our protein of interest with ^{35}S. Cell growth was slow in the sulfate-free minimal labelling medium. To increase cell yield initial growth was carried out in rich medium, the cells were harvested aseptically, and resuspended in the labelling medium.

The cells were first grown in 1.5 litres of LB containing 0.2% succinate to an OD_{600} of 0.9 (cuvette path length of 1 cm). Cells were harvested by centrifugation in sterile bottles, and were resuspended in 3 litres of M9 medium containing 1 mCi/l of [^{35}S]-SO_4 (New England

Nuclear). The cells were then allowed to grow to an OD_{600} of 0.8–0.9 (4 hours). About 85–90% of the $[^{35}S]$–SO_4 added was associated with the cells, and 4–5 g (wet weight) of cells were obtained. At the end of growth, the protease inhibitor PMSF (2% in absolute ethanol) was added to a final concentration of 0.002%. All of the subsequent solutions contain 0.002% PMSF. After centrifugation, the cell pellet was washed with 500 ml of 50 mM sodium phosphate buffer (pH 6.6). The washed pellet was resuspended in 40 volumes (i.e. 40 ml/g wet weight) of EDTA-sucrose solution, which was comprised of 20% sucrose, 1 mM EDTA, in 33 mM Tris-HCl (pH 7.2). After stirring the suspension for 15 minutes at room temperature, cells were harvested by centrifugation at 10,000 x g for 10 min at 4°C. This cell pellet was resuspended immediately in 40 volumes of ice cold 5 mM $MgCl_2$, stirred gently for 15 min at 4°C, and the cells were removed by centrifugation (10 min at 10,000 x g). The supernatants from both the EDTA-sucrose and osmotic shock treatments (containing the released proteins) were centrifuged again at 40,000 x g for 10 minutes to remove any particulate matter. Sodium phosphate buffer (1 M, pH 7.0) and EDTA (0.5 M, pH 7.0) were added to give a final concentration of 10 mM and 5 mM respectively. The solution was quickly frozen in dry ice–acetone, and stored at minus 20°C. To prevent nonspecific adsorption to glass and plastic surfaces, all the tubes and bottles were coated with dichloro-dimethyl silane (section 2.2.11).

2.2.5 Fractionation by Affinity Chromatography

A modification of the previously reported (46,58) procedure was used in the isolation. These modifications consisted of special precautions taken to minimize the extent of proteolytic degradation.

The isolation was carried out at room temperature because preliminary experiments had indicated that binding to the column was reduced at 4°C (T.C.Y. Lo, personal communication).

The frozen osmotic shock fluid was thawed quickly in a 37°C water bath, centrifuged 10 min at 40.000 x g to remove precipitated protein, and loaded onto aspartate-Sepharose columns (1.5 x 20 cm) equilibrated with 10 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 0.002% PMSF. Each column was loaded with 30-35 ml of shock fluid, and washed with two column volumes of the running buffer. Protein bound to the aspartate-Sepharose was eluted with 200 mM sodium succinate in the running buffer, and was collected into silane coated test tubes kept in ice. The elution profile was monitored by its radioactivity. The peak fraction ("succinate eluted protein" (SEP)) was pooled and desalted by passage through a Bio-Gel P-60G (Bio-Rad Inc.) column in 20 mM sodium phosphate (pH 7.0). The desalted SEP was aliquotted into silanized microfuge tubes (Eppendorf), frozen in dry ice-acetone, and stored at -20°C.

2.2.6 Electrophoresis of the Succinate Eluted Protein (SEP)

The purity of the isolated [^{35}S]-SEP was assessed using discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (59). The SEP was treated with 2% SDS in the presence of 2-mercaptoethanol for 5 min at 100°C. After running, the gels were either stained with Coomassie blue or sliced to determine radioactivity. 2.5 mm tube gels were cut into 3 mm slices using a wire form and the slices were dissolved in 1.0 ml of 30% H_2O_2 overnight at 65°C in tightly capped glass scintillation vials. Nine millilitres of liquid scintillation fluid was added and the vials were

counted in a liquid scintillation counter (Beckman Instruments).

Isoelectric focusing gel electrophoresis (IEF) was performed according to the method of the first dimension of O'Farrell (60). Urea was not used and the osmotic strength was maintained using sucrose. The tube gels were sliced as described and the radioactivity in the slices determined. The pH profile was determined by allowing each slice to equilibrate overnight in a tightly sealed vial containing 0.5 ml of degassed deionized water, and measuring the pH.

2.2.7 Transport Studies

For transport studies, cells (CBT43) were grown with vigorous shaking at 37°C overnight in LB medium. Cells were harvested by centrifugation, resuspended in fresh LB medium, and then allowed to grow for two hours. The culture was harvested, washed twice with 50 mM potassium phosphate buffer (pH 7.5), and resuspended in the buffer to an OD_{600} equivalent to 4.0.

Transport studies were initiated by the addition of 1 ml of the radioactive substrate in 50 mM phosphate buffer (pH 7.5) to 1 ml of cells suspended in the same buffer. The final cell concentration was equivalent to an OD_{600} of 0.4 (3.5×10^8 cells/ml). Purified nonradioactive SEP (isolated from the osmotic shock fluid) was added to the cells immediately before the addition of the substrate, as required. Transport studies were carried out at room temperature with stirring. Samples (0.2 ml) were taken at 30 sec intervals, filtered through 0.45 μm pore size nitrocellulose membrane filters, and washed with 10 ml of ice cold phosphate buffer. Filters were counted in 5 ml of scintillation fluid in a liquid scintillation counter.

2.2.8 HPLC/Gel Filtration

For determination of molecular weight by HPLC/gel filtration, an LKB Ultropac TSK G3000 SW column (7.5 x 600 mm) was used. The running buffer contained 50 mM Na_2SO_4, 10 mM NaH_2PO_4, and 0.05% sodium azide, pH 7.4. Approximately 10 µg of SEP in 10–20 µl of running buffer was injected onto the column. The flow rate was 1 ml/min. The elution profile was monitored by absorbance at 280 nm and samples were collected at 1 min intervals to determine radioactivity.

2.2.9 Amino Acid Analysis

Protein and peptide samples were hydrolyzed in 6 N HCl in evacuated sealed tubes for 18 hr at 115°C, dried using a Buchler Flash-Evaporator, and dissolved in 0.2 M sodium citrate buffer, pH 2.2. The hydrolyzates were analyzed on a Beckman Auto-Programmed 119CL single column analyzer using the techniques and conditions outlined in the instruction manual. Peaks were identified and quantitated by comparison with a calibration mixture. Cysteine was determined as cysteic acid following performic acid oxidation (61). Since histidine was eluted where the change occurred between buffer 2 and 3, histidine was analyzed separately using only buffer 3 as the running buffer.

The N-terminal residue was determined by dansylation (62), followed by acid hydrolysis, and identification of the dansylated residues by thin layer chromatography on polyamide plates (63).

2.2.10 Peptide Mapping By HPLC

SEP was digested by trypsin at 37°C for 6 hrs in 50 mM ammonium bicarbonate (pH 8.0); the SEP:enzyme ratio was 50:1 (64). After digestion, the peptides generated were analyzed by reverse phase HPLC chromatography. A Waters µBondapak C_{18} column was used in these

studies. The gradient used to elute the peptides was from 0% to 60% acetonitrile, containing 0.05% trifluoroacetic acid (TFA) The flow rate was 1 ml/min.

2.2.11 Silanization of Tubes

The lids and insides of microfuge tubes were treated with a 5% solution of dichloro-dimethyl silane (Eastman) in chloroform. The $CHCl_3$ was removed by heating the tubes in a 65° oven for at least 30 min. The tubes were washed 3-4 times with tap water, rinsed in deionized water, and dried in at 65°. Collection tubes, pipette tips and storage tubes were treated in a similar fashion.

2.3 RESULTS

2.3.1 Purification of Succinate Eluted Protein (SEP)

The succinate eluted protein (SEP)), was isolated by a modification of the procedure reported (46,58) earlier for the purification of DBP. These procedural changes consisted of addition of protease inhibitors and rapid freezing of protein solutions. Fig. 2.1 shows the elution profile of SEP from the aspartate-Sepharose column upon addition of succinate. Fig. 2.1A is the elution profile of EDTA-sucrose releasable material, Fig. 2.1B shows the elution profile of osmotic shock releasable proteins. The purity of the osmotic shock SEP was determined by SDS-PAGE and isoelectric focusing gels (Fig. 2.2 and Fig. 2.4A ("+ mercaptoethanol")). Using radioactively labelled SEP the radioactive peak was found to coincide with the protein band stained by Coomassie blue. By comparison with the molecular weight standards the molecular weight of SEP was determined to be around 56K. A protein, isolated from the EDTA-sucrose releasable material, had the same molecular weight

FIGURE 2.1

ELUTION PROFILES OF SEP ON ASPARTATE-SEPHAROSE

Proteins released by various treatments from [^{35}S]-labelled CBT43 were loaded onto aspartate-Sepharose columns. The columns were equilibrated with 10 mM sodium phosphate (pH 7.0) containing 5 mM EDTA, and 0.002% PMSF. Peak I represents proteins which were not retained by the column. Peak II represents the succinate eluted protein (SEP). SEP was eluted with 200 mM succinate in the running buffer.

(A) Elution profile of proteins released by EDTA-sucrose treatment.

(B) Elution profile of proteins released by osmotic shocking.

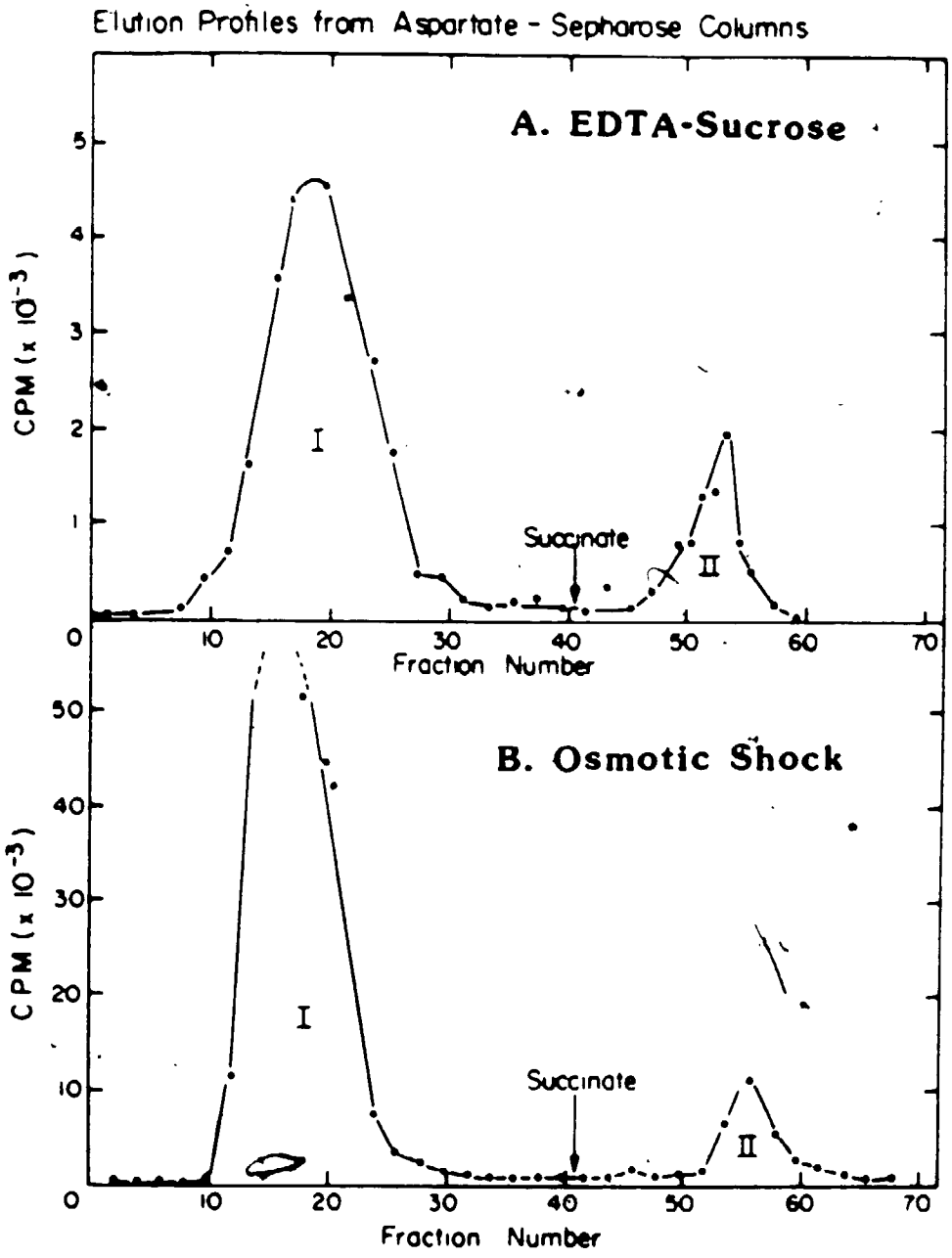

Elution Profiles from Aspartate - Sepharose Columns
A. EDTA-Sucrose
B. Osmotic Shock
CPM (x 10⁻³)
Fraction Number
Succinate
I
II
0
10
20
30
40
50
60
70
1
2
3
4
5
10
20
30
40
50

FIGURE 2.2

GEL ELECTROPHORESIS OF [^{35}S]-SEP

(A) and (B): SDS-PAGE gel electrophoresis was performed according to Laemmli (59). 2-Mercaptoethanol denatured SEP from the aspartate-Sepharose affinity columns was run on 2.5 mm tube gels. Following electrophoresis the tube gel was sliced into 3 mm slices, the slices were solubilized in H_2O_2 and the radioactivity counted as described in "Experimental Procedures".

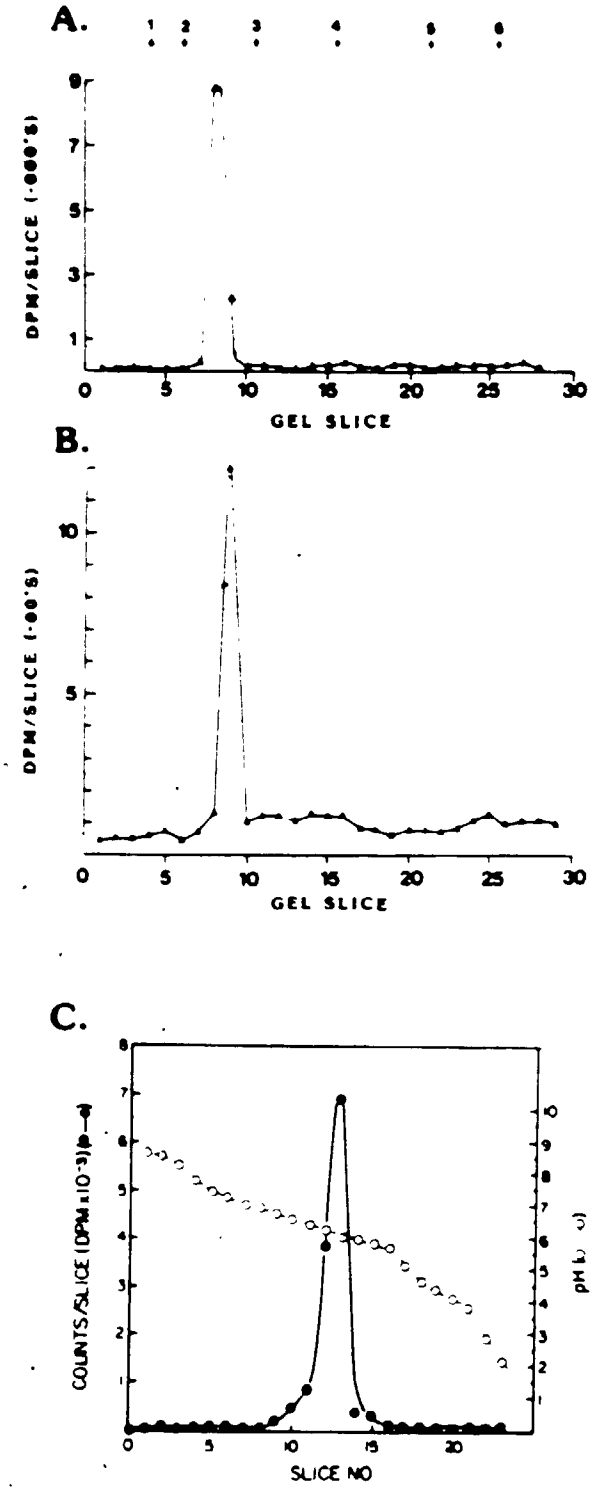
(A) Osmotic shock SEP.

The arrows indicate the position of standards (Pharmacia): 1, phosphorylase B (94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000); 5, soya bean trypsin inhibitor (20,100); 6, lactalbumin (14,400). These standards had been labelled with ^{125}I.

(B) EDTA-sucrose SEP.

(C) Isoelectric focusing gel (IEF) of osmotic shock SEP.

IEF tube gels were run according to the first dimension of O'Farrell (60) in the absence of urea as described in "Experimental Procedures". The gels were sliced and the radioactivity counted (●) or the pH determined (○).


A.
DPM/SLICE (·000'S)
GEL SLICE
B.
DPM/SLICE (·00'S)
GEL SLICE
C.
COUNTS/SLICE (DPM x 10⁻³)
SLICE NO

(Fig. 2.2B). It was apparent from these analyses that SEP was free from other contaminating radioactive proteins. The calibration curve of the isoelectric focusing gel indicates that SEP had an isoelectric pH value of around 6.2. (Fig. 2.2C).

Because of the apparent similarity of the SEP isolated from EDTA-sucrose and osmotic shock releasable fractions, and due to the much larger amount of protein that can be isolated from the osmotic shock, all of the following characterizations were carried out with osmotic shock SEP and this will generally be referred to as "SEP".

2.3.2 Stimulation of Succinate Transport Activity Upon Addition of Purified SEP

It was postulated earlier that the cell surface DBP acts as the substrate recognition component for the nonspecific porin transport channel (45,47), and that the transport capacity can be increased by the addition of purified DBP to whole cells (45). Thus the biological activity of the purified SEP can be easily assessed by its effect on the whole cell transport activity. Fig. 2.3 shows that the addition of purified osmotic shock SEP to intact wild type cells (CBT43) resulted in an increase in succinate transport activity. This increase was not due to contaminating bacteria because no succinate transport activity was seen in the presence of SEP alone (i.e in the absence of cells). These results indicate that SEP had a biological activity similar to that previously reported by Bewick and Lo (45) for DBP.

2.3.3 Altered Mobility of SEP Under Non-Reduced Conditions

When SEP was run on SDS-PAGE in the absence of 2-mercaptoethanol (Fig 2.4A and B) a decreased mobility (i.e. an apparently higher molecular weight) was seen. Even a slight amount of 2-mercaptoethanol,

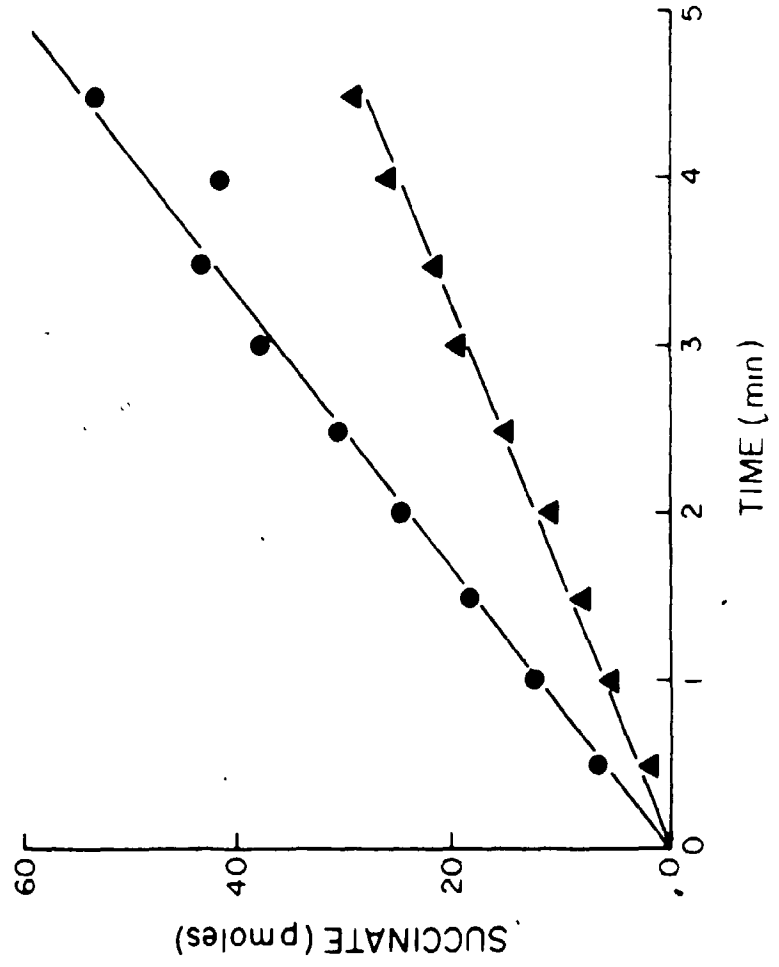
FIGURE 2.3

ACTIVATION OF SUCCINATE TRANSPORT BY PURIFIED SEP

Transport studies were carried out as outlined in "Experimental Procedures". The final concentration of succinate was 2 μM; the specific activity of the succinate was 1,000 DPM/3.8 pmoles.

(▲) - Transport in the absence of SEP.

(●) - Transport in the presence of osmotic shock SEP.


SUCCINATE (pmoles)
60
40
20
0
0
1
2
3
4
5
TIME (min)

diffusing across the gel gave the lower molecular weight in at least part of the sample lane (Fig. 2.4A, right hand lane). As seen in Fig. 2.4B molecular weight standards either had an unaltered mobility or ran "faster" (e.g. "B"-BSA, and "O"-ovalbumin) in the absence of 2-mercaptoethanol.

Figure 2.4C shows the SDS-PAGE profiles of the proteins released by osmotic shock (from M9-grown CBT43) in the presence or absence of 2-mercaptoethanol. It is apparent that two major proteins, 52K and 56K are present in this preparation. Based on the response of the purified SEP and the 56K protein in the crude extract to the mercaptoethanol treatment, it seems likely that SEP is a major protein released by osmotic shock treatment.

It can be seen from Fig. 2.4C that, in the absence of 2-mercaptoethanol, the majority of the osmotic shock releasable proteins migrated at the same position or with a smaller apparent molecular weight. The unusual behaviour of SEP stands out, especially in comparison to the other major protein at 52K.

A similar decrease in the apparent molecular weight of SEP was seen in the presence of dithiothreitol, indicating this was a general response to reducing agents. Boiling was not required to elicit the change. Boiling the protein in the absence of reducing agent did not change the protein's slower migration, indicating that denaturation was probably not important for the molecular weight shift.

2.3.4 HPLC/Gel Filtration Properties of Purified SEP

The molecular weight of the native form of SEP was determined by high-pressure liquid chromatography (HPLC) gel filtration. An LKB Ultropac TSK G3000 column was used. This gel filtration column

FIGURE 2.4

EFFECT OF 2-MERCAPTOETHANOL ON MIGRATION OF SEP IN SDS-PAGE GELS

(A) Pure SEP in the presence (+) or absence (-) of 2-mercaptoethanol.

The left side of the SDS-PAGE gel contains standards and osmotic shock SEP run in the presence of 2-mercaptoethanol and the right side is SEP run in its absence. The far right lane contained sample buffer with 2-mercaptoethanol present, run adjacent to a lane containing SEP in the absence of 2-mercaptoethanol. 'S' indicates the position of SEP. The identity of the molecular weight markers (Pharmacia) are outlined in Figure 2.1A.

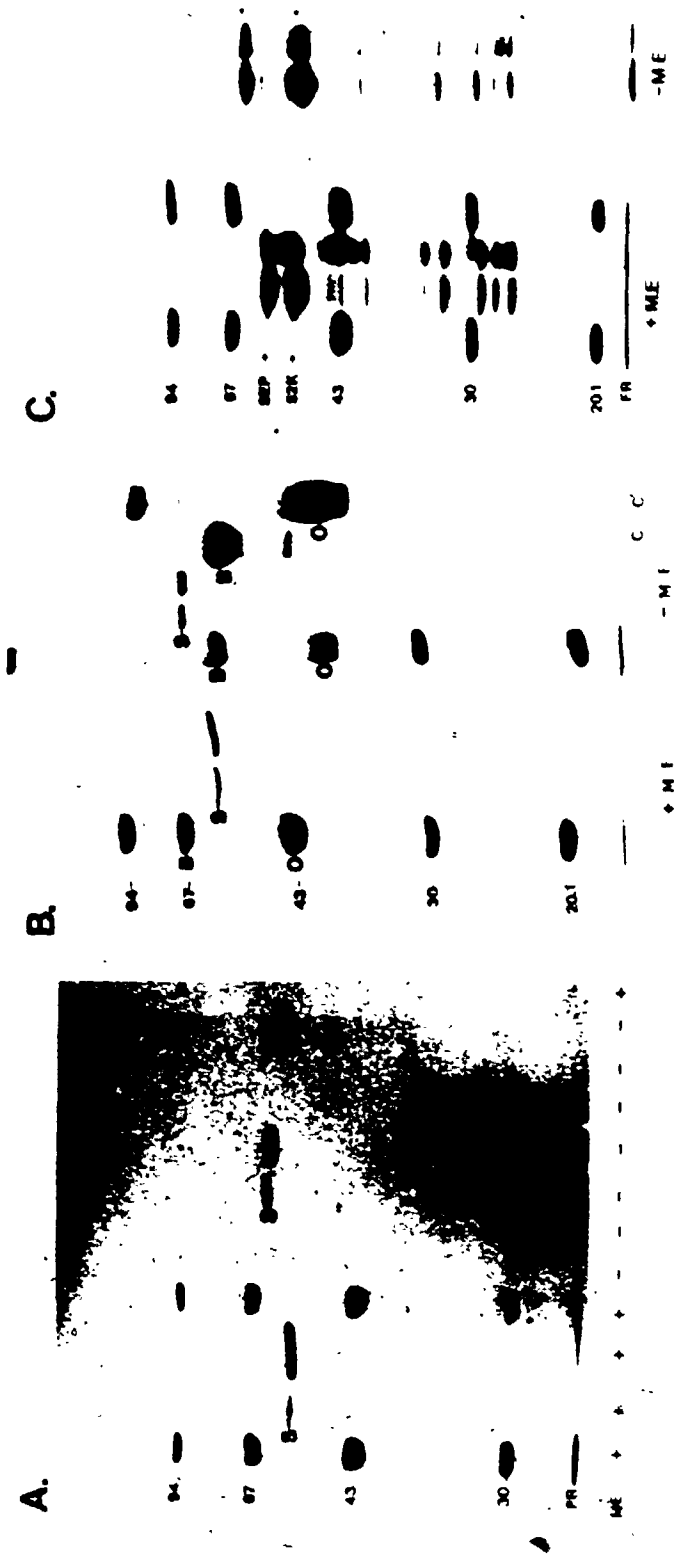
(B) Comparison of SEP and molecular weight standards on SDS-PAGE gels in the presence or absence of 2-mercaptoethanol.

The left side of the gel contains standards and osmotic shock SEP run under reducing conditions. The right side of the gel contains standards and SEP run in the absence of mercaptoethanol. C and C' are commercially available BSA (Sigma) and ovalbumin (Pharmacia) respectively, whose position among the standards is indicated by 'B' and 'O'. S indicates the position of SEP.

(C) SDS-PAGE of Total Osmotic Shock Releasable Proteins in the Presence or Absence of 2-Mercaptoethanol.

M9-grown CBT43 were osmotically shocked and the proteins released were run on SDS-PAGE in the presence (left side) or absence (right side) of 2-mercaptoethanol, and compared to mercaptoethanol reduced standards. Approximately 30 µg of protein was loaded onto the gel. The positions of SEP and the other major osmotic shock releasable protein at 52K are marked.

All gels were stained with Coomassie blue.


A.
B.
C.
-ME
+ME
-MI
+MI
FR
30
43
67
94
201

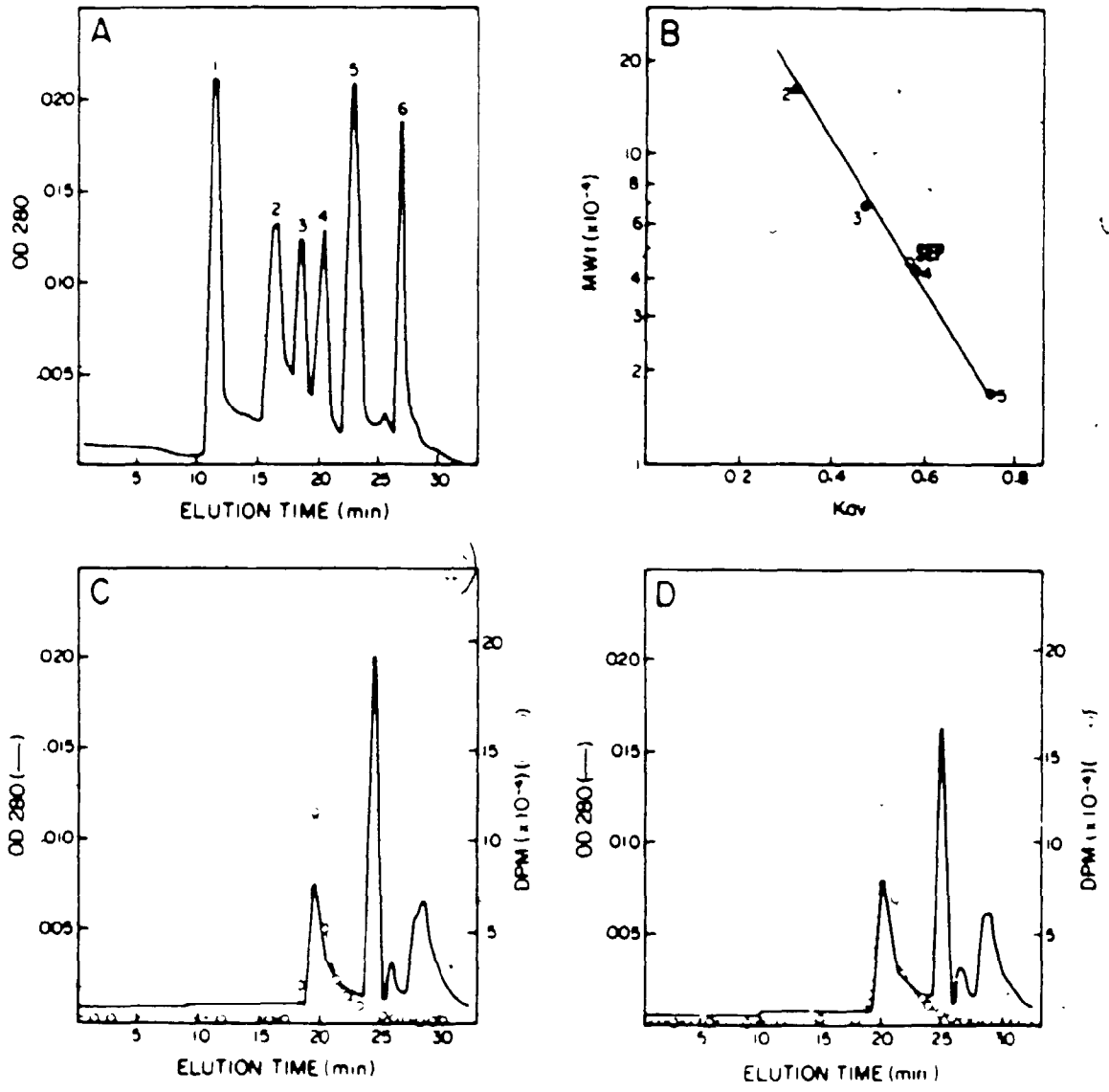
separates proteins on the basis of effective size in aqueous solution, and has a molecular weight exclusion limit of 480,000 for proteins. Fig. 2.5 shows the elution profiles of various protein standards (Fig. 2.5A) and SEP. Using radioactively labelled SEP, the radioactive peak was found to coincide with a 280 nm absorbance peak (Figs. 2.5C and 2.5D). From the calibration curve established with various protein standards (Fig. 2.5B), the molecular weight of SEP was determined to be around 45K; just slightly larger than ovalbumin. This was different from the molecular weight determined by SDS-PAGE. SEP and ovalbumin migrated quite differently on SDS-PAGE gels especially under non-denatured conditions. The apparent reduction in molecular weight as determined by HPLC may be due to retardation of the elution of SEP from the column. This may be attributed to the shape of SEP, or to nonspecific adsorption (i.e. by ionic and electrostatic interactions) to the column. Since the silica based support material has been shown to act as a weak cation exchanger (65), it is possible that the movement of SEP through the column may be hindered by nonspecific interaction. It is apparent from this study that SEP normally occurs in a monomeric form; subunit aggregates cannot be detected under this non-denaturing albeit <u>in vitro</u> condition. Fig. 2.5D shows that 250 uM succinate had no effect on the elution profile. Thus succinate, the substrate for DBP, had no apparent effect on the conformation or aggregation of SEP.

Both Figs. 2.5C and 2.5D show that several small molecular weight 280 nm absorbance peaks were present in the SEP sample. Judging from the radioactive peaks, and Coomassie blue stained protein bands in SDS-PAGE and isoelectric focusing gels, and from the radioactive peak eluted

FIGURE 2.5

MOLECULAR WEIGHT OF SEP AS DETERMINED BY HPLC/GEL FILTRATION

An LKB Ultropac TSK G3000 SW column (7.5 x 600 mm) was used to determine the molecular weight of SEP. The running buffer contained 50 mM Na_2SO_4, 10 mM NaH_2PO_4, and 0.05% sodium azide, pH 7.4. Fig 2.5A shows the elution profile of the calibration mixture. 1, blue dextran (>2,000,000); 2, gamma-globulin (160,000); 3, bovine serum albumin (67,000); 4, ovalbumin (43,000); 5, myoglobin (17,000); and tyrosine (180). Fig. 2.5B shows the molecular weight calibration curve as determined by the markers used in 2.5A. The molecular weight of SEP is indicated by the open circle. Fig 2.5C shows the elution profile of radioactive SEP. The open circles indicate the radioactivity of the various fractions. The identity of the low molecular weight species is discussed in the text. Fig. 2.5D shows the elution profile of radioactive SEP in the presence of 250 μM sodium succinate (pH 7.4). Fractions were also counted for radioactivity.


A
OD 280
ELUTION TIME (min)
B
MW (x10⁻⁴)
Kav
C
OD 280 (—)
DPM (x10⁻⁴)
ELUTION TIME (min)
D
OD 280 (—)
DPM (x10⁻⁴)
ELUTION TIME (min)

from the gel filtration column, we were confident that the SEP samples were free of any detectable contaminating proteins. The identity of the smaller molecular weight UV absorbing material is unclear. It should be mentioned that during purification of SEP, the passage of isolated SEP through the Bio-Gel P-60G column removed some small molecular weight compounds, which have an absorbance peak around 257 nm. These will be discussed below (Section 2.3.7). It is possible that the low molecular weight compounds observed in Figs. 2.5C and 2.5D may be residual A_{257} factors still associated with SEP after passage through the P-60G column.

2.3.5 Amino Acid Composition of SEP

The amino acid composition of SEP was determined as shown in Table 2.1. When SEP is compared to a number of other proteins with a molecular weight similar to SEP (e.g. E. coli lipoamide dehydrogenase (56K), human plasma α_1, α_2 globulin (57K), and chicken or bovine glutamine dehydrogenase (56K) (66)) the protein appears to have higher levels of proline, tyrosine, and threonine residues, but lower levels of glycine, while the other residues are not that different. Similar to other substrate binding proteins (67), SEP contains a low percentage of cysteine (0.3%) and methionine (1.4%).

Using SEP prepared from cells grown in the presence of $[^{35}S]$-SO_4, 74% of the radioactivity was found to be associated with cysteine (2 residues) and the rest with methionine (6 residues). Thus it seems cysteine residues were preferentially labelled in $[^{35}S]$-SO_4 grown cells.

N-terminal analysis revealed a dominant spot at the position of dansyl glycine and a faint spot at the position of dansyl-alanine.

TABLE 2.1

AMINO ACID COMPOSITION OF SEP
AND OTHER PROTEINS OF SIMILAR MOLECULAR WEIGHT

AMINO ACID	(MOLE %)	RESIDUES / 56K	Residues/Molecular Weight a	b	c	d
GLY	6.3	31	58	59	47	48
ALA	8.2	41	59	32	37	40
VAL	7.7	38	49	23	34	33
LEU	8.2	41	38	25	31	32
ILE	4.0	20	42	11	37	35
PRO	6.1	30	26	21	21	20
SER	6.2	31	19	64	30	28
THR	7.6	38	28	24	28	27
ASx	12.3	61	45	34	50	48
GLx	10.5	52	53	62	45	48
Half-CYS	0.3	2	2	n.d.	6	7
MET	1.3	6	10	6	13	15
LYS	7.9	39	41	15	33	35
ARG	3.5	17	16	12	30	28
HIS	2.0	10	13	22	14	16
PHE	2.5	12	16	22	23	23
TYR	5.6	28	10	12	18	17
TRP	n.d.		n.d.	30	3	3

n.d. - Not Determined

The amino acid composition of SEP was determined as outlined in "Experimental Procedures". The amino acid composition of the other proteins was from Ref. 66 and are provided for comparison. The identity of the other proteins are: (a) *E. coli* lipoamide dehydrogenase (56K); (b)Human plasma α_1, α_2 globulin (57K); (c) Bovine glutamate dehydrogenase (55K); Chicken glutamate dehydrogenase (56K)

2.3.6 Peptide Map of SEP

SEP tryptic peptides were analysed by reverse phase HPLC. An acetonitrile gradient was used to elute the peptides. Fig. 2.6 shows that at least 15 major peptides can be resolved. Using SEP from [^{35}S]-SO_4 grown cells, radioactivity can be detected in peptides 2, 5, 11, 13, and 14. By determining the number of counts associated with each peak it was calculated that 5%, 10%, 31%, 49%, and 5% of the total radioactivity was associated with these peptides respectively.

In view of the distribution of ^{35}S counts in cysteine and methionihe (section 2.3.5) and the number of these residues per molecule (Table 2.1), it can be calculated that each cysteine and methionine residue accounts for around 35% and 5% of the total radioactivity respectively. Based on this calculation, it can be inferred that one cysteine residue was present in peptides 11 and 13; one methionine residue was present in peptides 2, and 14; and peptides 5 and 13 both contain two methionine residues. Amino acid analysis of the peptides indicated that one cysteine residue could be detected in peptides 11 and 13. It should be mentioned that the above calculation was based on the assumption that peptide 12 did not contain cysteine or methionine, it was possible that some of the radioactivity associated with peptide 13 might be due to the presence of these residues in peptide 12.

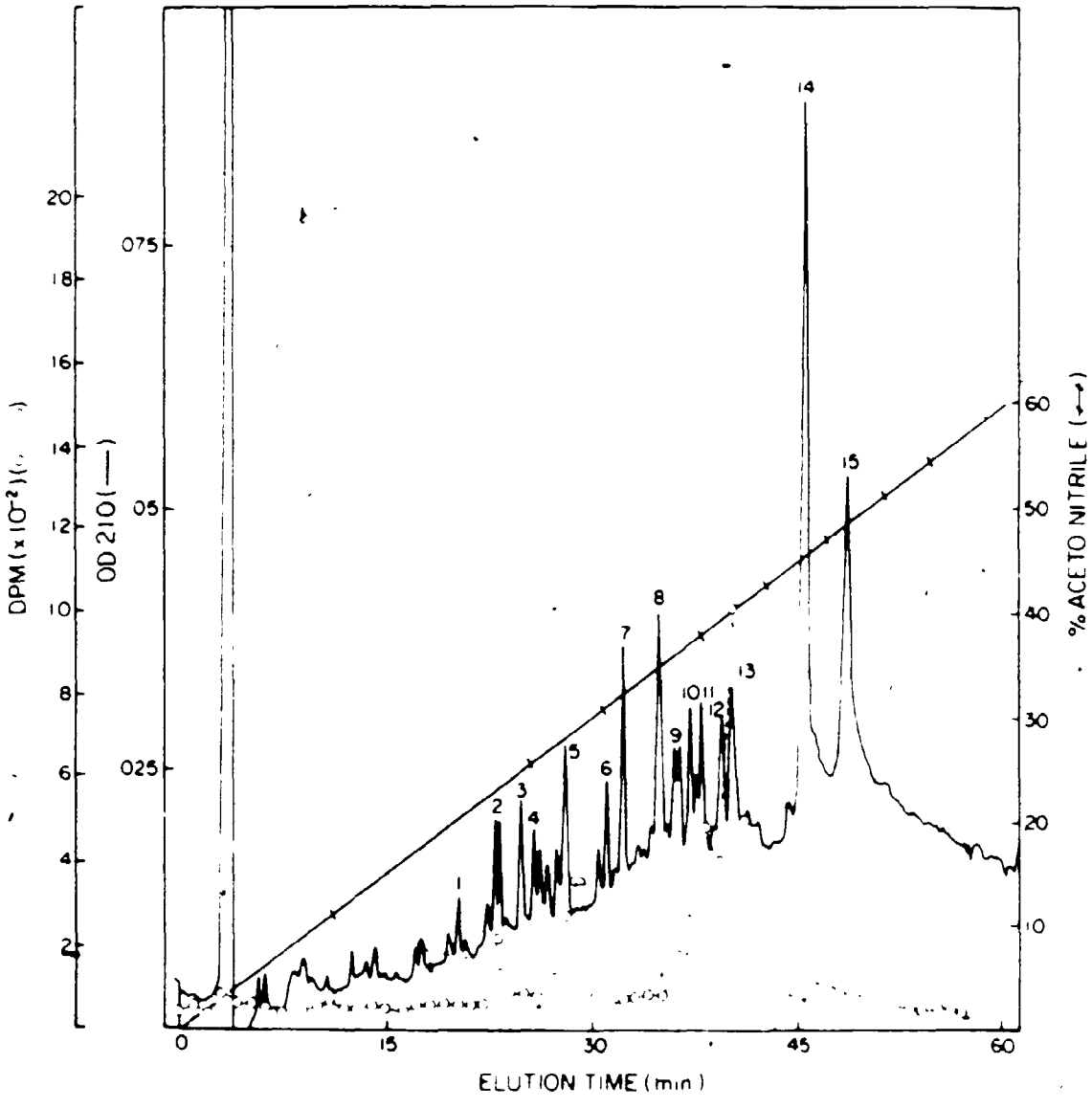
2.3.7 Associated Material Eluting with SEP

The EDTA-sucrose releasable and osmotic shock fluid from E. coli contains a considerable amount of material with an absorption maximum at 257 nm. This absorption is close to what would be expected for nucleotides, but the osmotic shock was done under conditions that minimize release of cytoplasmic contents (1 mM EDTA in the EDTA-sucrose,

FIGURE 2.6

HPLC PEPTIDE MAP OF SEP

Peptides generated by tryptic digestion of [^{35}S]-labelled SEP were analyzed by reverse phase HPLC on a Waters uBondapak C_{18} column. The gradient used was from 0 to 60% acetonitrile, in 0.05% TFA, at 1 ml/min. In order to detect peptides containing the [^{35}S]-labelled cysteine and methionine, fractions were collected and counted for radioactivity (○).


DPM (x10⁻²)
OD 210 (—)
% ACETONITRILE (⟶)
ELUTION TIME (min)
0
15
30
45
60
075
05
025
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

and 5 mM $MgCl_2$ in the osmotic shock (Ref. 68 and Chapter 3). The protein eluted by succinate from the aspartate–Sepharose column also had a 257 nm absorbance maximum even though the columns were washed with at least 2 column volumes of buffer before elution. This indicates that this material (referred to as the "DK factor" or DKF) was either associated with the SEP or was binding to the column and co-eluting with it. Following elution from the aspartate–Sepharose column, SEP was passed through Bio-Gel P-6DG desalting gel to remove succinate. This column also separated the DKF from the SEP. The DKF eluted after the succinate peak and very much later than the protein (Fig. 2.7). This indicates that the DKF was probably sticking to the P-6DG column which is reported to have properties of low adsorptivity (69).

Following P-6DG gel filtration the SEP appeared to be free of DKF as the absorption peak of the protein was now close to 280 nm (see Fig. 4.1B), similar to other proteins. The disruption of the SEP-DKF interaction on the P-6DG may be due to the presence of succinate, or due to a preferential association of DKF with the gel matrix.

A large amount of A_{257} material could be separated on P-6DG before loading onto aspartate–Sepharose. When pre-adsorbed osmotic shock fluid was loaded onto aspartate–Sepharose, and the succinate eluted peak run through another P-6DG column, there was a noticeable reduction in the DKF in comparison to non-P-6DG pretreated (Fig. 2.8).

2.3.8 DKF is Labelled with Uridine

As DKF had an absorption maximum similar to nucleotides, attempts were made to label the compound with thymidine and uridine. For this purpose cells were grown in M9 in the presence of [^{3}H]-thymidine or

FIGURE 2.7

ELUTION PROFILE OF SEP ON BIO-GEL P-6DG

[^{35}S]-SEP isolated by 200 mM succinate elution of aspartate-Sepharose was passed through Bio-Gel P-6DG to remove succinate. Collection of fractions was begun after the sample had been loaded onto the column. The position of protein was monitored by its radioactivity (●). 100 µl aliquots of the fractions were counted.

The position of succinate was monitored by its conductivity (□).

The position of the "DK-factor" (DKF) was monitored by its absorbance at 257 nm (△).

FIGURE 2.8

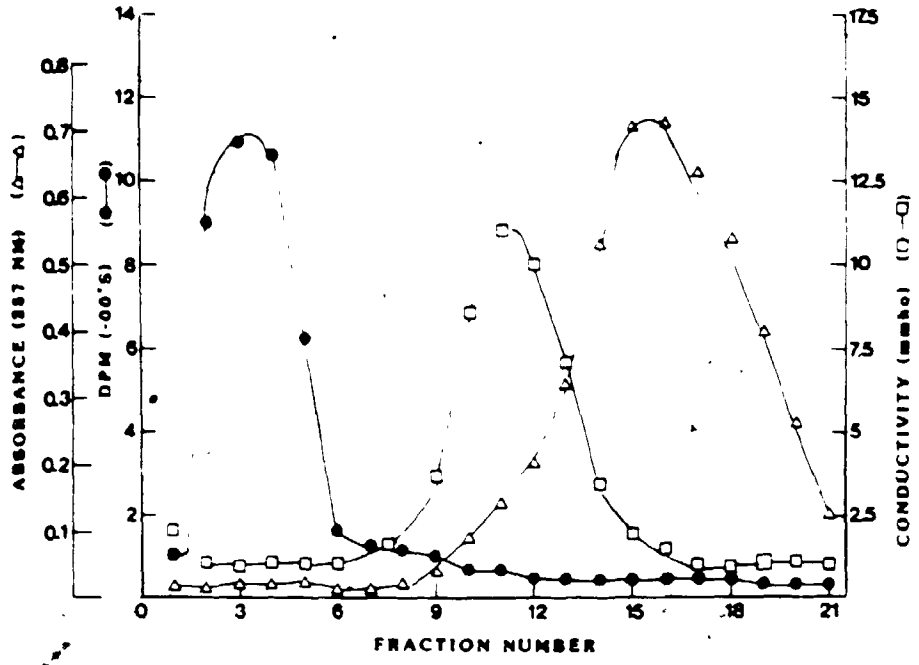
REMOVAL OF DKF FROM SEP BY BIO-GEL P-6DG PRETREATMENT

Osmotic shock fluid from CBT43 was passed through a column of Bio-Gel P-6DG. The early eluting material (λ_{max} = 274 nm) was passed through aspartate-Sepharose to isolate SEP. The SEP was then run on a second column of P-6DG. This figure shows the elution profile from the second P-6DG column.

The position of succinate is indicated by its conductivity (△).

The amount of DKF present was monitored by its absorbance at 257 nm.

(○) - Pre-Aspartate-Sepharose P-6DG pretreated
(●) - No pretreatment


ABSORBANCE (257 NM) (Δ—Δ)
DPM (-00'S) (●—●)
CONDUCTIVITY (mmho) (□—□)
FRACTION NUMBER

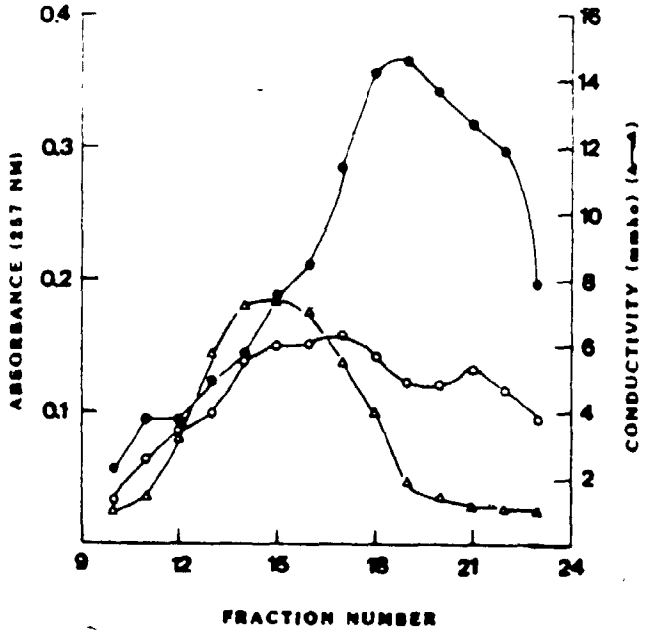

ABSORBANCE (257 NM)
CONDUCTIVITY (mmho) (Δ—Δ)
FRACTION NUMBER

FIGURE 2.9

INCORPORATION OF THYMIDINE AND URIDINE INTO DKF

CBT43 had been grown in M9 in the presence of [^{3}H]-thymidine or [^{3}H]-uridine. The osmotic shock fluid was isolated and passed through aspartate-Sepharose columns (A and B). The succinate eluted peak was then passed through columns of Bio-Gel P-6DG. In all of these columns absorbance at 257 nm, conductivity, and radioactivity was monitored.

(A-B) Aspartate-Sepharose chromatography

The columns were loaded with osmotic shock fluid and washed with 2 column volumes of buffer. 200 mM succinate was used to elute bound proteins. The succinate was added at Fraction 1.

(A) Osmotic shock fluid from [^{3}H]-Thymidine grown CBT43
(B) Osmotic shock fluid from [^{3}H]-Uridine grown CBT43

(C-D) Bio-Gel P-6DG Gel Filtration

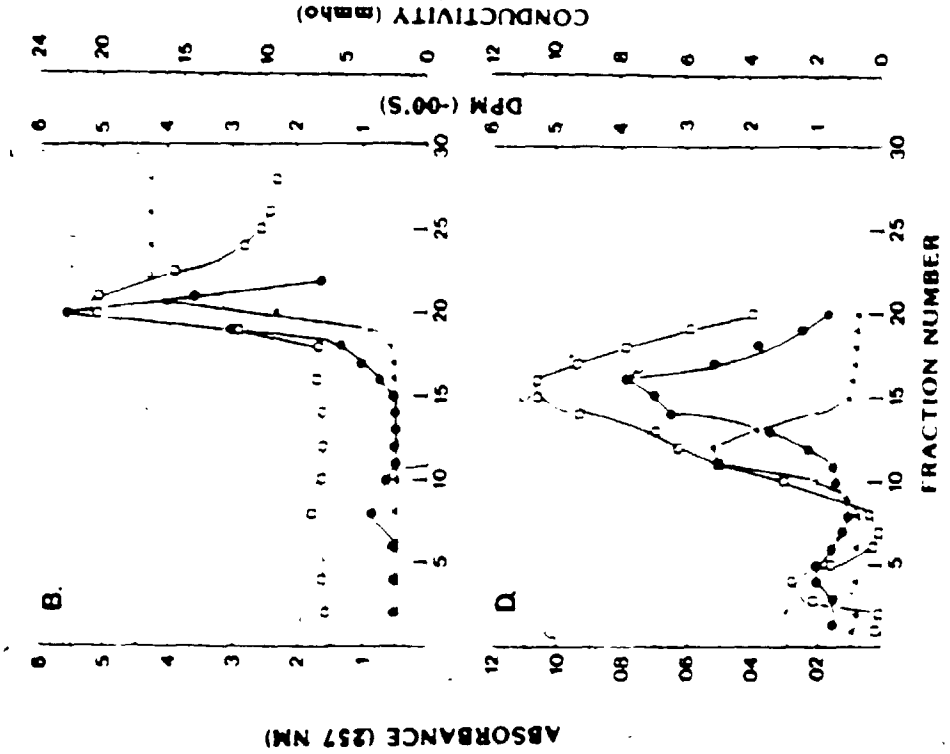
Collection of samples was begun after the sample had loaded. The column was equilibrated with 20 mM phosphate buffer, pH 7.0.

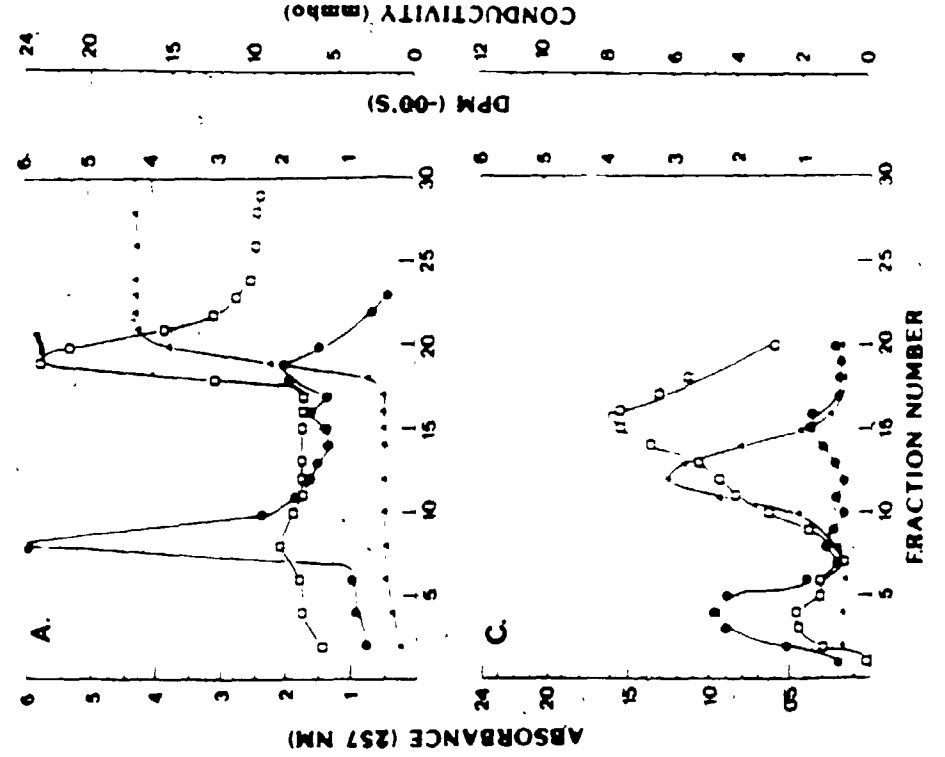
(C) Eluted material from [^{3}H]-Thymidine labelled cells
(D) Eluted material from [^{3}H]-Uridine labelled cells

(●) - Radioactivity (DPM/100 ul aliquot)

(□) - Absorbance (257 nm)

(△) - Conductivity (mmho)


CONDUCTIVITY (mmho)
DPM (-00'S)
B.
D.
FRACTION NUMBER
ABSORBANCE (257 NM)


CONDUCTIVITY (mmho)
DPM (-00'S)
A.
C.
FRACTION NUMBER
ABSORBANCE (257 NM)

[^{3}H]-uridine. The osmotic shock fluid was passed through aspartate-Sepharose (Figs. 2.9A and 2.9B) and the succinate eluted peak passed through Bio-Gel P-60G (Figs. 2.9C and 2.9D). It can be seen (Figs. 2.9B and 2.9D) that [^{3}H]-uridine labelled a product with elution characteristics similar to DKF. On the other hand, radioactivity from [^{3}H]-thymidine was primarily found in a compound eluting with the protein from P-60G (Figs. 2.9A and 2.9C).

2.4 DISCUSSION

The initial studies by Lo and Sanwal implicated DBP in the transport of dicarboxylic acids across the cell envelope of Escherichia coli (44). A molecular weight of 16K was reported. Further studies by Bewick and Lo localized DBP to the cell surface as well as the periplasmic space (44,57). In these later studies DBP was identified based on its ability to bind to aspartate-Sepharose.

We originally wished to isolate DBP in a pure form and study its physicochemical properties. To prevent degradation of the protein during isolation we used proteolytic inhibitors (0.002% PMSF, and 5 mM EDTA), but otherwise ran the aspartate-Sepharose columns according to Bewick (46,58) (i.e. in 10 mM sodium phosphate). It should be mentioned that DBP was originally isolated by Lo using buffer containing 50 mM phosphate and 50 mM arsenate (44,57). The columns were run at room temperature, and the collected fractions were kept in ice after elution.

The other change implemented was to osmotically shock the cells into cold 5 mM $MgCl_2$ (instead of H_2O) according to the method of Nossal and Heppel (68). These researchers showed that shocking logarithmic phase cells into cold water could cause lysis and release cytoplasmic components. The importance of Mg^{++} in the shock procedure will also be

shown in Chapter 3.

Using these techniques, a single protein species was eluted from the column with succinate. This protein will be referred to as the "succinate eluted protein" (SEP). The molecular weight of SEP was determined by SDS-PAGE to be around 56K.

Addition of SEP to intact whole cells resulted in an increase in succinate transport activity. This is similar to what was previously seen (45) and indicates that we may be looking at a protein similar to that was isolated by Bewick (45,58). On the other hand, SEP may be quite different from the DBP originally reported by Lo as SEP has a much larger molecular weight and is eluted from the aspartate-Sepharose by relatively low ionic strength buffers (see Chapter 3).

A single 56K protein species was isolated from both EDTA-sucrose releasable and osmotic shock releasable fractions. In view of the greater amount of SEP releasable by the osmotic shock treatment, this population of protein was used for the physicochemical studies. Isoelectric focusing and HPLC/gel filtration studies also revealed the presence of only a single radioactively labelled protein species. SEP exhibited an unusual behaviour on SDS-PAGE in the absence of 2-mercaptoethanol, in that it had an apparently higher molecular weight. It was shown that purified SEP had a similar molecular weight, and responded similarly to mercaptoethanol, as one of the major proteins released by osmotic shock. It may be surmised from this observation that SEP may indeed be the 56K major cell envelope protein released by osmotic shock treatment.

2-mercaptoethanol is thought to act by reducing intrachain

disulfide bonds, causing an unfolding of the α-helix, so that long spindle shaped molecules are formed in the presence of SDS (70). This unfolding retards the rate of migration through the gel. Oligomeric proteins joined by interchain disulfides will run at a higher molecular weight in the absence of reducing agents. The change in molecular weight seen here was too small to be due to the disruption of a homo-oligomer. A hetero-oligomer with a small molecular weight subunit is another possibility. As the proteins should be uniformly labelled with ^{35}S, the presence of a cysteine-containing small molecular weight subunit should have been visible in autoradiograms (especially in light of the preferential labelling of cysteine residues shown below). No small molecular weight subunit was detected in autoradiograms of fixed and stained gels or a gel that was dried and autoradiographed immediately after running. As well, no significant difference in the intensity of the SEP band was noted in the presence or absence of 2-mercaptoethanol. This unusual behavior under non-reducing conditions may be explained in a number of ways. A hetero-oligomer may be present but the small subunit may be too small or with insufficient [^{35}S]-labelled residues to be detectable. Alternately, the reducing agents may alter the protein in such a way that it binds with more SDS and hence runs faster, or there may be an interaction between the acrylamide matrix and the protein that is disrupted by 2-mercaptoethanol.

Smaller molecular weight factors that were not labelled with ^{35}S were observed in the HPLC/gel filtration profile. This may be the residual "DK-Factor" still associated with the protein. This "DKF" co-eluted with SEP from the aspartate-Sepharose column, had an absorbance maximum around 257 nm, and could be separated from protein with a Bio-

Gel P-6DG desalting column. On this column the DKF was eluted after the salt peak. The DKF was found to be labelled by [^{3}H]-uridine.

Amino acid analysis of SEP indicated that the protein had low levels of cysteine and methionine residues, in common with other binding proteins (67). Analysis of [^{35}S]-labelled SEP revealed that about 74% of the radioactivity resided in the cysteine residues. About 15 major peptides could be identified in the tryptic digest of SEP. Peptide mapping of the radioactive SEP indicated that cysteine residues might reside in peptides 11 and 13.

In summary, this chapter reports the isolation of a 56K major cell envelope protein from E. coli K12. This protein was found to be able to activate succinate transport when added externally. Detailed analyses of the physicochemical properties of this protein were also carried out.

CHAPTER 3

OPTIMIZATION OF SEP PRODUCTION, RELEASE, AND ISOLATION

3.1 INTRODUCTION

In the preceeding chapter the preliminary isolation and physicochemical identification of the SEP was outlined. The SEP functionally resembled the protein isolated by Bewick (45,58) in that both proteins were able to increase succinate uptake in whole cells when added externally.

To further characterize SEP we decided it was necessary to optimize the isolation procedure and to establish the location of this protein in the cell envelope. Given its ability to stimulate succinate uptake, SEP was assumed to be involved in dicarboxylate transport, and thus, the preliminary step in the optimization involved the determination of growth conditions at which succinate uptake was maximal. Using cells grown under the optimal conditions, we then established conditions for the osmotic shock procedure that would provide maximal release of periplasmic proteins with minimal leakage of cytoplasmic contents. We used the release by EDTA and lysozyme ("spheroplasting"), in conjunction with osmotic shocking to confirm the cell envelope location of SEP.

The last stage in the optimization involved determination of aspartate–Sepharose running conditions which would provide the maximum recovery of SEP with minimal contamination by other proteins.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 Bacterial Strains and Growth Medium

All the optimization studies were carried out with the *E. coli* K12 strain CBT43. The composition of LB and M9 were given in the previous

chapter. Cells grown in M9 were started from a 1:10 inoculum of overnight LB grown cells.

3.2.2 Transport Assays

Transport assays were carried out with 2 uM succinate as outlined in the previous chapter. In the case of the growth stage dependence studies, the procedure for harvesting and washing the cells was modified. Cells were filtered through 0.45 μM nitrocellulose filters (Nucleopore), washed twice with 5 ml of Hepes-KOH (50 mM, pH 7.5) and resuspended in this buffer to an OD_{600} of 0.8. Uptake was initiated by adding 4 μM [^{14}C]-succinate (New England Nuclear, carrier free, 1000 dpm/9.45 pmoles) to an equal volume of cell suspension.

3.2.3 Release of SEP From Cells

3.2.3.1 Osmotic Shock - Lysozyme Treatment

EDTA-sucrose and osmotic shock treatments of the cells to release cell surface and periplasmic contents were carried out as outlined in section 2.2.4 except where noted in the text. Following osmotic shock, a lysozyme treatment was often carried out in an attempt to isolate periplasmic proteins "bound to" or "retained by" the peptidoglycan network (46). After osmotic shock the cell pellet was incubated in "sucrose-Tris" (20% sucrose, 33 mM Tris-HCl, pH 7.2) for 10-15 min and then lysozyme (Sigma) (0.1 mg/ml) and EDTA (1 mM) were added. After 10-15 min with slow stirring at room temperature, the cells were spun down at 6,000 x g for 10 min at 20°C. To remove remaining particulate matter, the supernatant, containing the "lysozyme releasable" proteins, was centrifuged at 40,000 x g for 10 min at 4°C.

3.2.3.2 Spheroplast Formation

The preparation of spheroplasts was carried out essentially as

outlined by Osborne and Munson (53). After growth, cells were harvested, washed once with 50 mM sodium phosphate (pH 7.2), and resuspended in 40 volumes (i.e. 40 ml/gm wet weight) of room temperature sucrose-Tris. After 10 min lysozyme and EDTA were added to give 0.25 mg/ml and 2 mM respectively. After 10-15 min at room temperature, centrifugation was carried out as in section 3.2.3.1. and the supernatant, containing the "spheroplast releasable" proteins was saved. The success of the spheroplast formation procedure was assessed by diluting treated cells 1:10 into distilled water and determining whether a plasmolysis dependent decrease in OD_{600} was occurring.

3.2.3.3 Small Scale Osmotic Shock Treatment

To study SDS-PAGE profiles of released proteins under a variety of conditions, a small scale isolation procedure was implemented. Fifteen ml of overnight grown LB was used to inoculate 150 ml of succinate-glycerol-LB supplemented M9. The cells were grown for 4 hrs, washed with 100 ml of 50 mM sodium phosphate, pH 7.2, and resuspended in 15 ml of sucrose-Tris. 1.0 ml aliquots were put in 1.5 ml microfuge tubes, EDTA was added to the desired concentration, and the cells were left to stand at room temperature for 10 min. The cells were spun in a microcentrifuge (11,000 x g) for 3 min, and the supernatant was removed and respun for 7 min. This second supernatant was transferred to a new microfuge tube and TCA was added to a final concentration of 10%. Protein precipitation was carried out for at least 1 hour on ice. The TCA precipitated protein was centrifuged for 10 min and the pellet was washed twice with diethyl ether. Ether was removed by evaporation in a 65°C oven, and the pellet was dissolved in Laemmli sample buffer.

Residual acid, its presence indicated by change in colour of the sample buffer, was neutralized with ul quantities of dilute NaOH.

The cell pellet from the EDTA-sucrose treatment was resuspended in the residual medium, the hypo-osmotic shocking solution (ice-cold 5 mM $MgCl_2$, unless otherwise noted) was added, and the mixture was vortexed for 1-3 sec. The tubes were then allowed to sit on ice for 10 min, spun in the microfuge and the supernatant was treated as above.

3.2.4 Assay of β-galactosidase Activity

β-galactosidase, a cytoplasmic enzyme, was used as a marker to determine release of cytoplasmic contents during the various release procedures. β-galactosidase was present in the cells even in the absence of the usual inducer isopropyl-1-thio-β-D-galactopyranoside (IPTG). The LB medium probably contains a sufficient level of a natural inducer (e.g. lactose) to induce the synthesis of the enzyme. (M9 contains 1% fresh LB and cells were started from a 1:10 inoculation of LB grown cells). After growth the cells were washed in 50 mM sodium phosphate (pH 7.0) and either subjected to the release treatments or resuspended in phosphate buffer for sonication.

β-galactosidase was assayed as described by Miller (5). The reaction was carried out in Z-buffer (pH 7.0) which consisted of 60 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 50 mM mercaptoethanol. The substrate was o-nitrophenyl-β-D-galactoside (ONPG, Sigma). 200 µl of 4 mg ONPG/ml (in H_2O) was added to 1.2 ml of protein (in Z-buffer). The reaction was carried out for 15 min at 32°C, and stopped by the addition of 600 µl of 0.5 M sodium bicarbonate. The A_{410} of the mixture was then determined.

3.2.5 Aspartate–Sepharose Chromatography

Osmotic shock fluid (5 mM $MgCl_2$) was obtained from M9 grown cells that had been grown for 4.5 hr. The osmotic shock fluid was stored frozen (−20°C) until used (usually within 2-3 weeks). Concentrated phosphate and EDTA were added after thawing and prior to centrifugation and loading. PMSF (0.002%) was present in the original osmotic shock but owing to its short half-life in aqueous solutions an equivalent amount of fresh PMSF was added after thawing. All of the buffers contained 0.002% PMSF, added just prior to use on the columns. The aspartate–Sepharose columns were run as described previously, except where noted. Fractions from the elutions were TCA precipitated and prepared for SDS–PAGE as described above.

3.3 RESULTS

3.3.1 Effect of Growth Phase on Succinate Uptake

The change in the rate of succinate uptake was followed during growth to determine the growth phase at which the cells exhibited the maximum uptake. The succinate uptake rates were determined with 2 μM succinate, a concentration at which transport across the outer membrane should be rate limiting (47). Figure 3.1A indicates that, for LB grown cells, there was a sharp decrease in the uptake rates as the cells were shifted from stationary to logarithmic growth phase. The transport activity reached a minimum in early logarithmic stage, at about 90 min. The maximal value was attained in late log - early stationary phase after which it apparently remained high.

Quite a different profile was seen for M9 grown cells (Fig. 3.1B). Growth in M9 was much slower than in LB, but there was no initial

FIGURE 3.1

EFFECT OF GROWTH PHASE ON SUCCINATE UPTAKE

CBT43 grown in M9 or LB (containing 0.2% succinate) were assayed for succinate uptake ability at various times during growth. Aliquots were removed to measure growth and transport activity. Growth was monitored by change in optical density of the cultures at 600 nm. Uptake was measured at 2 uM (final) succinate as described in "Experimental Procedures". The rate of uptake was determined and is expressed as pmole succinate taken up per minute per 10^7 cells. Cultures consisted of 100 ml of cells in a 250 ml flask.

(A) Uptake in LB grown cells

(B) Uptake in M9 grown cells

(●) - Cell Growth (Absorbance at 600 nm)

(□) - Rate of Succinate Uptake (pmole x min^{-1} / 10^7 cells)

FIGURE 3.2

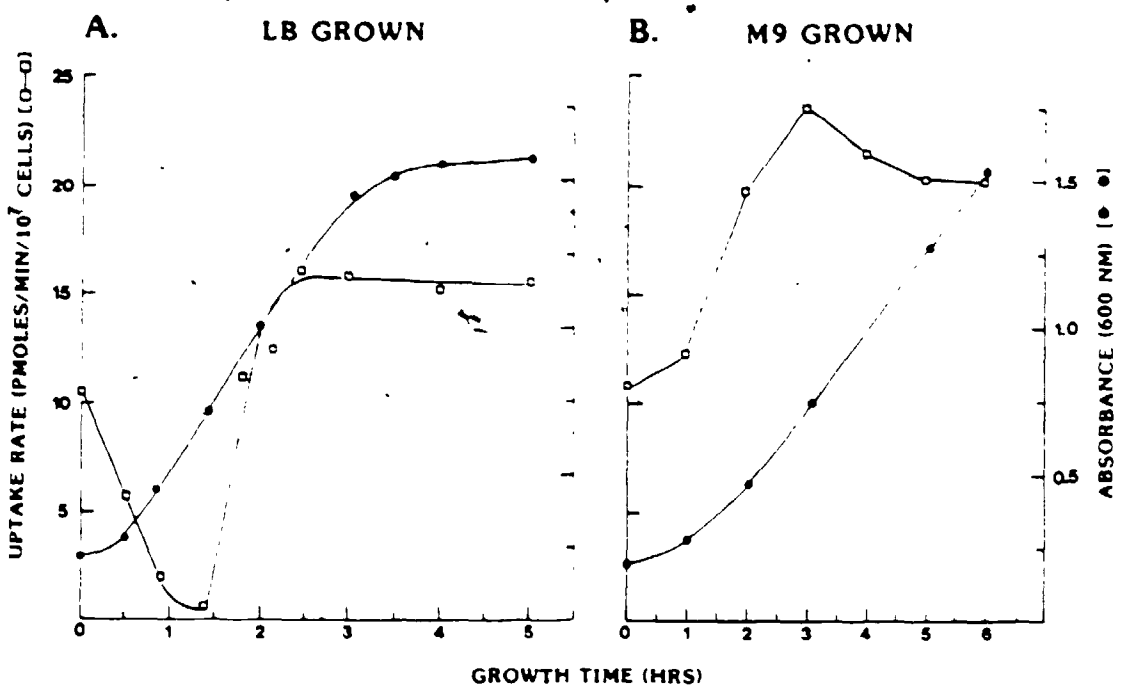
EFFECT OF CULTURE AERATION ON RATE OF SUCCINATE UPTAKE IN LB GROWN CELLS

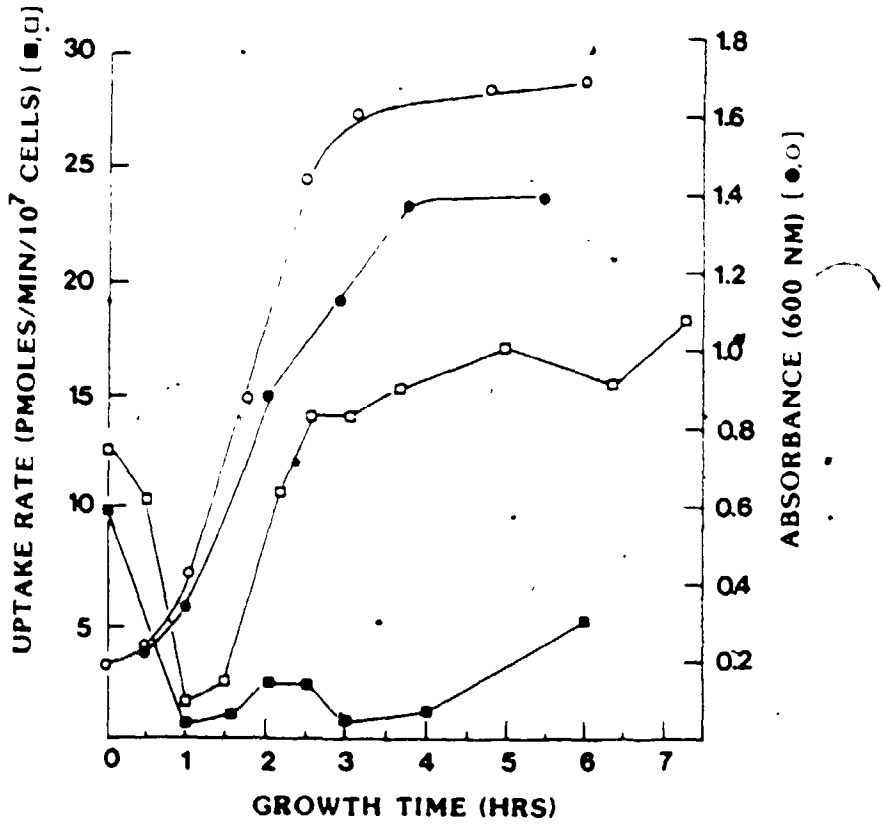
CBT43 was grown in LB (supplemented with 0.2% succinate). Cell growth and 2 uM succinate uptake were monitored. Growth was started from a 1:10 inoculum of overnight grown cells.

6 litre Erlenmeyer flasks (non-baffled) contained:
3 litres of LB and were shaken at 160 rpm in a New Brunswick Orbital Shaker (Low aeration, ●, ■)
or 2 l of LB at 200 rpm (High aeration, ○, □).

(●, ○) - Cell Growth (Absorbance at 600 nm)

(■, □) - Succinate Uptake Rate (pmole x min^{-1} / 10^7 cells)


A.
LB GROWN
B.
M9 GROWN
UPTAKE RATE (PMOLES/MIN/10⁷ CELLS) (○–○)
ABSORBANCE (600 NM) (● ●)
GROWTH TIME (HRS)


UPTAKE RATE (PMOLES/MIN/10⁷ CELLS) (■,□)
ABSORBANCE (600 NM) (●,○)
GROWTH TIME (HRS)

decrease in uptake rate as was seen in LB. Rather there seemed to be a gradual increase in transport activity early in the growth phase, after which it remained high. The levels of succinate uptake were at least 50% higher than the maximal level seen in LB grown cells. Kinetic analysis of the uptake indicated that the apparent K_m values of transport for LB and M9 grown cells were the same but M9 grown cells had higher V_{max} values. This suggests that the faster rate of transport might be due to an increased number of carriers or more efficient translocation across the cell envelope.

3.3.2 Effect of Culture Aeration on Succinate Uptake

The initial growth-stage experiments with LB-grown cells were performed on a small scale (100 ml in a 250 ml flask). When these experiments were scaled-up to a preparative scale (i.e. 3 litre in a 6 litre Erlenmeyer flask, in a New Brunswick Orbital Shaker with shaking at 160 rpm) quite a different profile was obtained (Fig. 3.2). The uptake rate for LB-grown cells did not increase until very late into the growth stage. To determine if this was due to insufficient aeration, 2 litres of cells were grown in 6 litre flask with shaking at 200 rpm. As can be seen in Fig. 3.2, there was a considerable increase in transport activity when the extent of aeration was increased. The profile resembled that obtained for the smaller scale assay, with maximal activity being reached after about 3 hours. The rate of succinate uptake at 3 hrs with 2 l cultures at high aeration was about 20 times the rate of uptake seen in the 3 litre cultures (low aeration) at the same time point.

3.3.3 Release of Protein from $^{35}SO_4$-Labelled E. coli

To determine the optimal conditions for protein release, $[^{35}S]$-SO_4

labelled cells were used. Material released under a range different conditions was TCA precipitated with 10% (final) TCA. The amount of radioactivity in the TCA precipitated material was taken as a measure of the protein released.

3.3.3.1 Effect of EDTA in EDTA-Sucrose

A range of EDTA concentrations from 0-5 mM was used in the EDTA-sucrose solution (containing 20% sucrose, 33 mM Tris-HCl (pH 7.2)). As shown in Fig. 3.3A, 0.5-1.0 mM EDTA was required for maximal release of protein. Above 1.0 mM EDTA there was only a slight increase in the counts that were released.

3.3.3.2 Effect of Mg^{++} in the Osmotic Shock

It can be seen (Fig. 3.3B) that as the concentration of $MgCl_2$ in the osmotic shock was increased, the TCA insoluble radioactivity (i.e. protein) decreased and levelled off above 1 mM $MgCl_2$.

From Fig. 3.3B it can be seen that increasing the concentration of EDTA in the EDTA-sucrose solution resulted in an increase in protein being released at low Mg^{++} concentrations. This effect was observed up to 0.5-1 mM EDTA. As will be seen later, very low $MgCl_2$ concentrations in the osmotic shock result in a extensive leakage of cytoplasmic contents. The results presented here suggest that the increasing initial EDTA concentrations may render the cytoplasmic membrane more fragile, resulting in a greater release of protein in the osmotic shock.

3.3.4 Methods for Assessing Leakage of Cytoplasmic Contents

To ensure that only cell envelope proteins were released by various treatments it was important to determine whether there was leakage of cytoplasmic contents occurring in the various release steps. For this

FIGURE 3.3

EFFECT OF EDTA AND $MgCl_2$ ON PROTEIN RELEASE FROM THE CELL ENVELOPE

CBT43 was grown in M9 in the presence of $[^{35}S]$-SO_4. EDTA-sucrose, osmotic shocking and postt-osmotic shock lysozyme treatments were carried out. The material released was TCA precipitated (with 10% TCA final). The TCA precipitable $[^{35}S]$-radioactivity represents protein released.

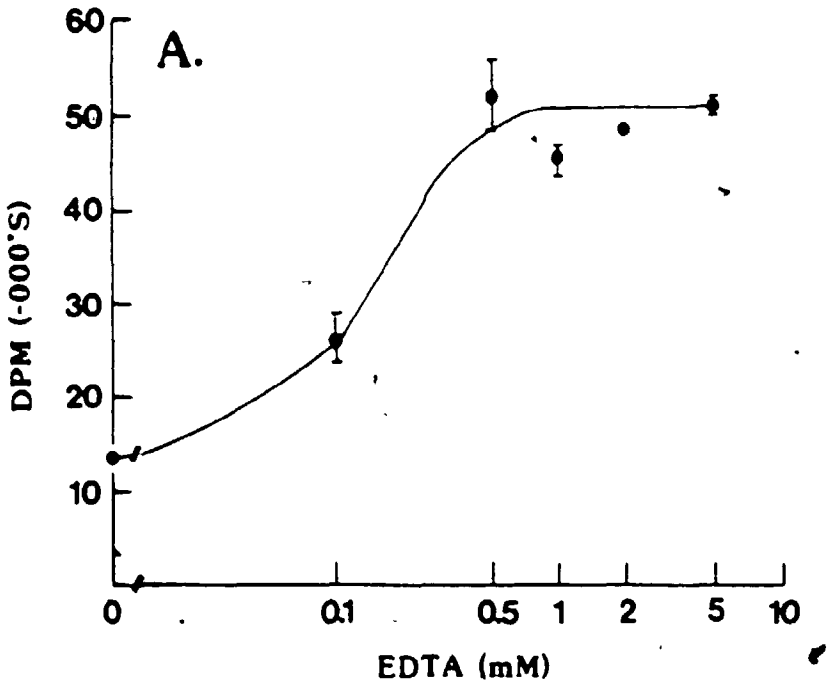
(A) Protein released by EDTA-sucrose treatments.

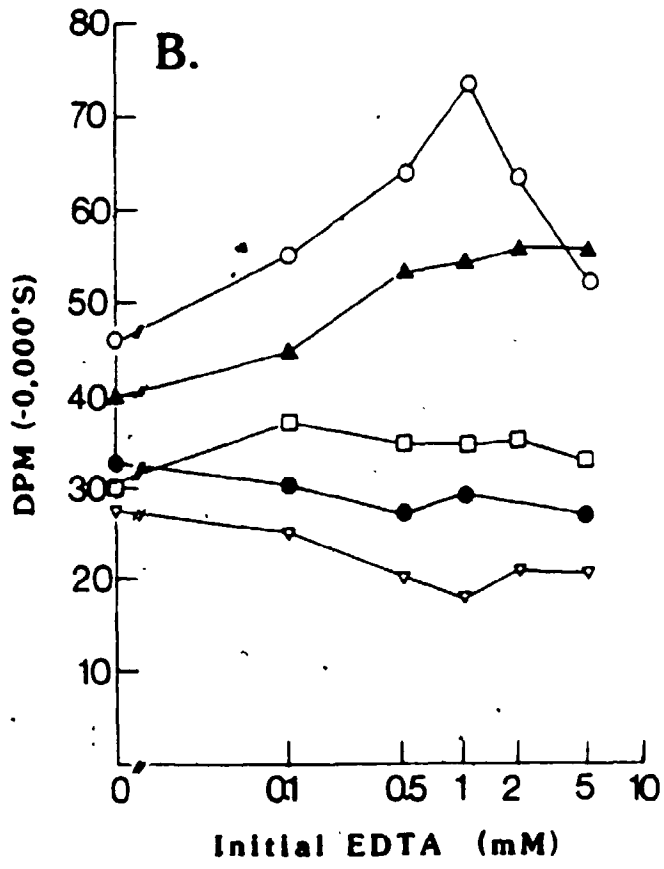
The TCA-insoluble material released with various concentrations of EDTA (0-5 mM) in the EDTA-sucrose was assayed.

(B) Protein released by osmotic shocking.

The concentration of $MgCl_2$ in the osmotic shock solution ranged from 0 to 10 mM. The results are plotted in terms of the initial EDTA-sucrose EDTA concentration. Each of the lines represents a $MgCl_2$ concentration.

(○) - 0 mM $MgCl_2$
(▲) - 0.1 mM $MgCl_2$
(□) - 0.5 mM $MgCl_2$
(●) - 1 mM $MgCl_2$
(▽) - 10 mM $MgCl_2$


A.
60
50
40
30
20
10
DPM (-000'S)
0
0.1
0.5
1
2
5
10
EDTA (mM)


B.
80
70
60
50
40
30
20
10
DPM (-0,000'S)
0
0.1
0.5
1
2
5
10
Initial EDTA (mM)

reason the amounts of DNA, RNA, and the cytoplasmic enzyme β-galactosidase released by the various fractionation procedures were quantitated.

3.3.4.1 Release of DNA

Cells were grown in the presence of [^{3}H]-thymidine to specifically label DNA. Macromolecules released by the various treatments were TCA precipitated. TCA should precipitate thymidine incorporated into DNA (presumably coming from lysed cells) but not free thymidine. 0.1 mM or 2 mM EDTA was used in the initial EDTA sucrose treatment. A small number of counts are released at each concentration in the form of free thymidine (Fig. 3.4A).

It can be seen from Fig. 3.6A that increasing the $MgCl_2$ concentration in the osmotic shock treatment caused a dramatic reduction in the TCA insoluble counts (i.e. DNA) released. At low $MgCl_2$ concentrations the majority of the released radioactivity was TCA-insoluble. At least 5 mM $MgCl_2$ was required to give a dramatic reduction in the recovered counts. The release of free thymidine (from a presumably cytoplasmic location) also decreased slightly with increasing $MgCl_2$ concentrations. The above findings indicate that cytoplasmic leakage was reduced or absent when the cells were shocked with 5 mM $MgCl_2$.

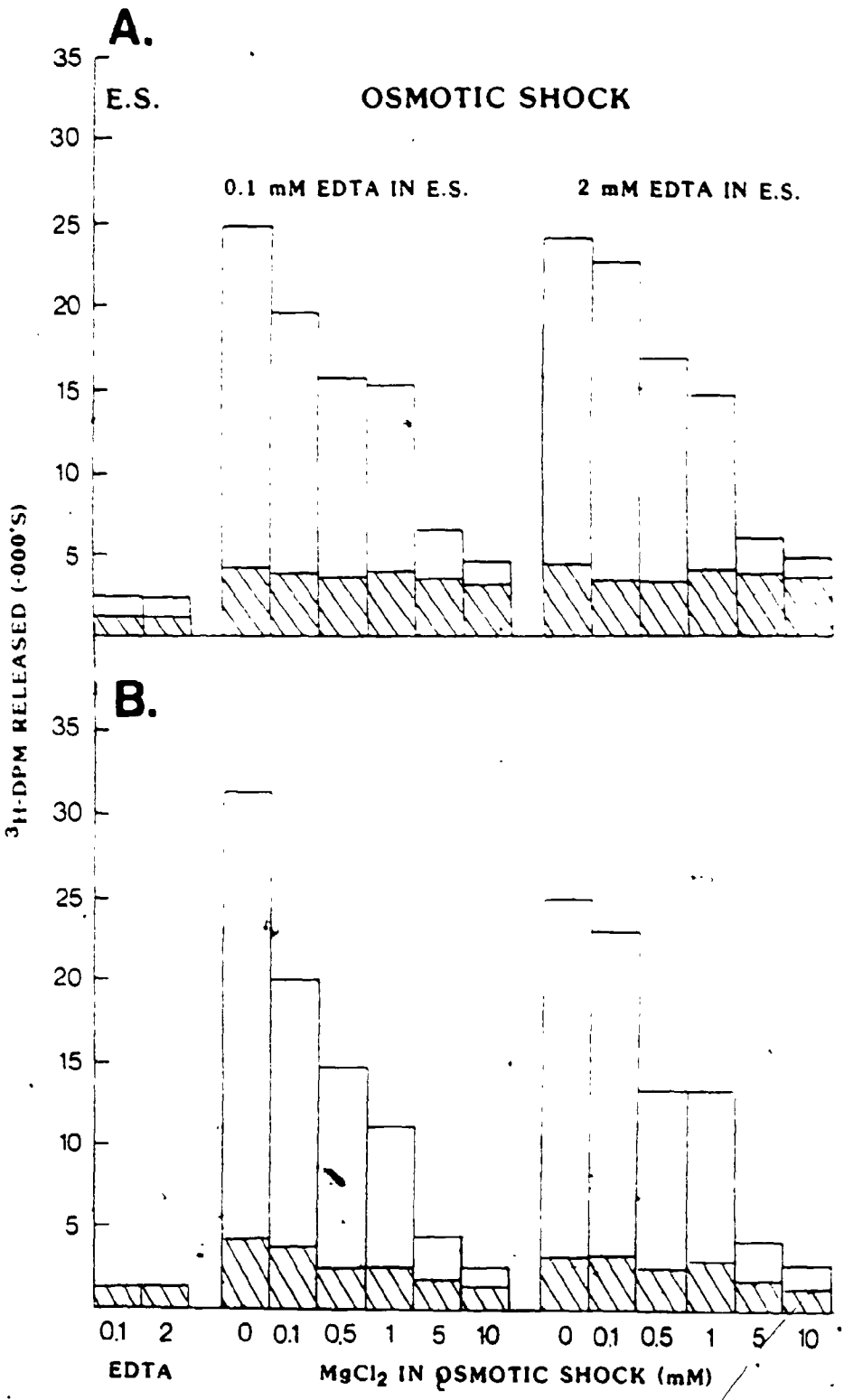
3.3.4.2 Release of RNA

To determine the extent of RNA leakage, cells were grown in the presence of [^{3}H]-uridine. The results (Fig. 3.4B) were very similar to those obtained for DNA leakage. Again, 5 mM $MgCl_2$ was required to minimize leakage of the TCA-insoluble material.

FIGURE 3.4

RELEASE OF DNA AND RNA BY OSMOTIC SHOCK TREATMENT

CBT43 was grown in M9 in the presence of (A) [^{3}H]-thymidine or (B) [^{3}H]-uridine to label DNA and RNA respectively. The cells were subjected to EDTA-sucrose and osmotic shock treatments. The released material was TCA precipitated with 15% (final) TCA. The EDTA-sucrose solution (E.S.) contained either 0.1 mM or 2 mM EDTA, while the concentration of $MgCl_2$ in the osmotic shock varied from 0 to 10 mM. The results are presented in terms of total [^{3}H]-counts released and TCA-soluble counts (hatched boxes). The difference (clear area) represents TCA-insoluble RNA or DNA.


A.
E.S.
OSMOTIC SHOCK
0.1 mM EDTA IN E.S.
2 mM EDTA IN E.S.
B.
^{3}H-DPM RELEASED (·000'S)
35
30
25
20
15
10
5
0.1 2
EDTA
0 0.1 0.5 1 5 10
0 0.1 0.5 1 5 10
MgCl$_2$ IN OSMOTIC SHOCK (mM)

3.3.4.3 Release of β-Galactosidase

β-galactosidase activity was also used to assess cell lysis. It can be seen from Table 3.1 that very little release of β-galactosidase occurred when the cells were treated with EDTA-sucrose (1%) or osmotically shocked into 5 mM $MgCl_2$ (0.9-1.2%). Decreasing the $MgCl_2$ concentration in the osmotic shock procedure below 1 mM lead to a considerable increase in β-galactosidase release. Eleven percent of the activity was released by shocking into cold water. Lysozyme treatment of osmotically shocked cells resulted in an almost complete release of β-galactosidase suggesting extensive cell lysis. This conclusion is corroborated by the large number of proteins seen in the "lysozyme releasable" fraction on SDS-PAGE that are different from those released by osmotic shock (i.e Fig. 3.12A, left hand side).

Formation of spheroplasts released a greater percentage of the β-galactosidase than was released by shocking into 5 mM $MgCl_2$, although 95% of the activity was still retained by the cells.

3.3.5 SDS-PAGE Profiles of Releasable Proteins

Proteins released under a variety of EDTA-sucrose and osmotic shock conditions from cells grown in M9 were examined by SDS-PAGE. This was a facile way to examine the release of SEP since it had been shown in the previous chapter that the 56K SEP was an easily identifiable major protein.

3.3.5.1 Effect of EDTA and pH

Increasing EDTA concentrations in the EDTA-sucrose (Fig. 3.5A) above 0.5 mM did not have any effect on the protein profiles seen on SDS-PAGE gels. Tris-sucrose itself caused release of protein (primarily 37K and 22K) but reduced release of the major 56K and 52K proteins was

TABLE 3.1

EFFECT OF CELL TREATMENTS ON RELEASE OF β-GALACTOSIDASE

	TREATMENT	PERCENTAGE RELEASED
	Sonicated cells	100
Experiment 1:		
	EDTA-sucrose	0.94
	Osmotic Shock	
	0 mM $MgCl_2$	11.3
	0.1 mM $MgCl_2$	8.6
	0.5 mM $MgCl_2$	5.4
	1 mM $MgCl_2$	3.3
	2 mM $MgCl_2$	1.9
	5 mM $MgCl_2$	1.2
	10 mM $MgCl_2$	1.2
Experiment 2:		
	EDTA-sucrose	0.87
	Osmotic Shock	
	5 mM $MgCl_2$	0.95
	Post-Osmotic Shock Lysozyme Release (0.01 mg/ml lysozyme)	
	0 mM EDTA	33
	1 mM EDTA	100
	2 mM EDTA	100
	5 mM EDTA	100
	Spheroplast Formation	5

Release of β-galactosidase activity (a cytoplasmic marker) from M9 grown log phase cells was measured to determine the extent of cytoplasmic leakage. Release treatments and assay of enzyme activity were as outlined in Section 3.2.4.

FIGURE 3.5

EFFECT OF EDTA-SUCROSE COMPOSITION ON THE PROTEINS RELEASED BY EDTA-SUCROSE AND OSMOTIC SHOCK TREATMENTS

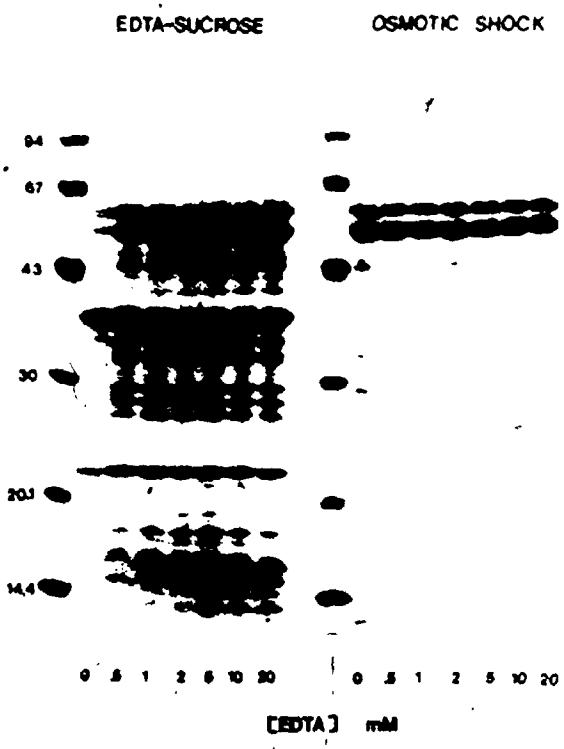
(A) Effect of EDTA concentration.

M9 grown CBT43 were subjected to an EDTA-sucrose treatment with concentrations of EDTA ranging from 0 to 20 mM. The pH was 7.2. The released proteins were 10% TCA precipitated, washed twice with ether, and solubilized in Laemmli sample buffer. The proteins were separated on SDS-PAGE gels and the gels stained with Coomassie blue. The treated cells were osmotically shocked into cold 5 mM $MgCl_2$, and the released proteins were treated similarly. The left side of the gel contains proteins released at the various EDTA concentrations, while the right side shows the proteins released in the subsequent osmotic shock step. Approximately 30 µg of protein was loaded in each lane. For the EDTA-sucrose samples the amount of protein loaded on is equivalent to that released from 20 OD_{600} units of cells. For the osmotic shock samples the amount of protein loaded on is equivalent to that released from 2.5 OD_{600} units of cells. (One OD_{600} unit is equal to 1 ml of cells with an OD_{600} of 1.0.)

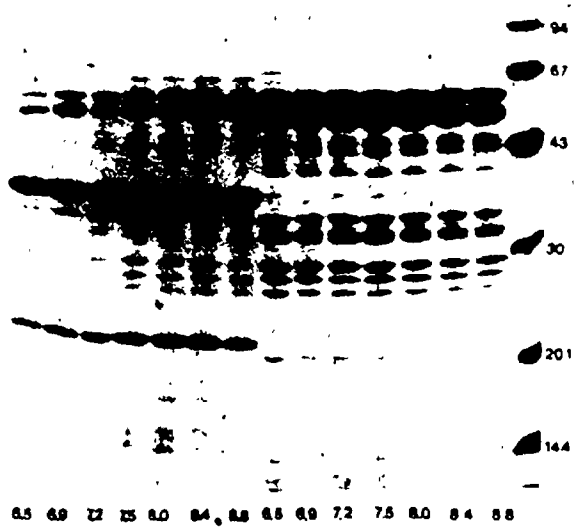
(B) Effect of pH of EDTA-sucrose on protein release.

M9-grown CBT43 were subjected to EDTA-sucrose treatments at various pH's. The concentration of EDTA was 2 mM. The treated cells were then subjected to a 5 mM $MgCl_2$ osmotic shock. The left side of the gel contains proteins released by EDTA-sucrose. The right side of the gel contains proteins released by osmotic shocking. The pH of the initial EDTA-sucrose treatment is indicated below each lane.

A. [EDTA] EFFECT



B. EFFECT OF TRIS pH



observed. The 37K and 21K proteins are most probably outer membrane proteins. The 37K protein was shown to react with antibodies prepared against OmpF (as outlined in section 5.2.6) and hence is one of the porins. When the cells were shocked into 5 mM $MgCl_2$ the 2 major proteins at 56K and 52K were present but the 37K and 22K proteins were absent. The initial EDTA concentration did not have a significant effect on the subsequent osmotic shock profiles.

The effect of the pH of the EDTA-sucrose treatment was tested in the presence of 2 mM EDTA (Fig. 3.5B). In this particular experiment there were smaller amounts of 56K and 52K proteins released by EDTA-sucrose. There was not much difference in the proteins released with increasing pH, although slightly greater levels seem to be present above pH 7.5. No change was seen in the proteins released in the subsequent 5 mM $MgCl_2$ osmotic shock. Because of this minimal difference, an EDTA-sucrose pH of 7.2 was selected as the method of choice.

3.3.5.2 Effect of Mg^{++} Concentration in the Osmotic Shock Treatment

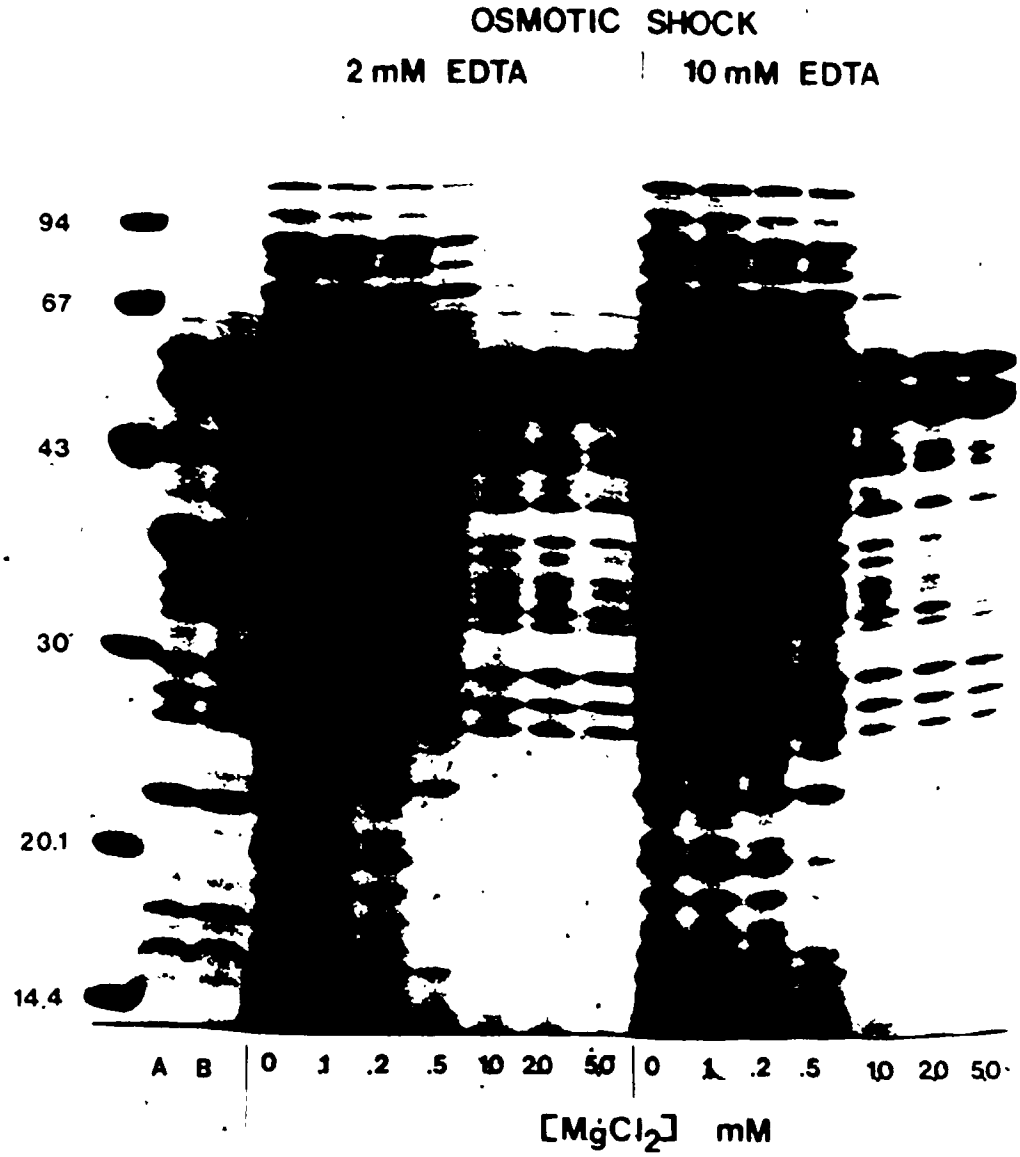
Cells were treated with EDTA-sucrose (pH 7.2) containing either 2 mM EDTA or 10 mM EDTA. The cells were then shocked into media containing 0 to 5 mM $MgCl_2$. It can be seen from Fig. 3.6, that a large number of proteins were released at low concentrations of $MgCl_2$. At higher concentrations of $MgCl_2$ the "typical" protein pattern was seen, with the 56K SEP and 52K being the major proteins. At low $MgCl_2$ concentrations major protein bands are located at 23K, 43K, 70K, and 86K. These were greatly reduced at higher concentrations of $MgCl_2$. The 43K protein may be the "major osmotic shock releasable protein" identified by Jacobson et al (52) as elongation factor Tu, a cytoplasmic

FIGURE 3.6

EFFECT OF MG^{++} CONCENTRATION IN OSMOTIC SHOCK ON PROTEIN RELEASE

M9 grown CBT43 were subjected to 2 or 10 mM EDTA in EDTA-sucrose (released proteins are in lanes A and B) and then subjected to osmotic shock into ice cold water containing 0 to 5 mM $MgCl_2$. The released proteins were treated as in the previous figures before being run on the SDS-PAGE gel. The gels were stained with Coomassie blue. The left side of the gel contains proteins released at the various $MgCl_2$ concentrations after initial 2 mM EDTA-sucrose. The right side contains proteins released after initial 10 mM EDTA-sucrose. The concentration of $MgCl_2$ is indicated below each lane. The amount of protein loaded was equivalent to that released from 2.5 OD_{600} units of cells.

EFFECT OF [$MgCl_2$] IN OSMOTIC SHOCK



protein of presumedly peripheral inner membrane location. The increased amount of protein released at low $MgCl_2$ concentrations is probably attributable to release of cytoplasmic contents as shown by release of DNA, RNA and β-galactosidase under similar conditions. The fact that the amount of released SEP remains relatively constant across the concentration range is an excellent indication of its cell envelope location.

3.3.6 Total Osmotic Shock Profiles Under Different Growth Conditions

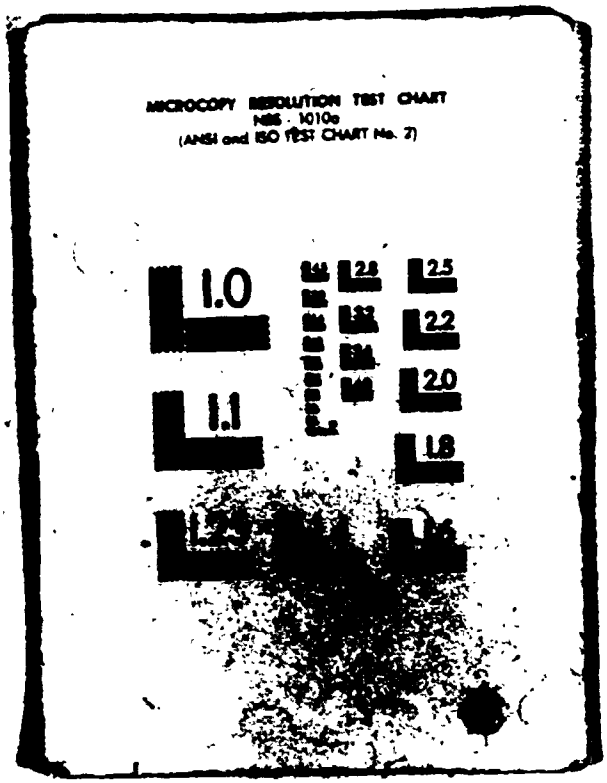
The osmotic shock releasable proteins from LB and M9 grown cells were examined by SDS-PAGE (Fig. 3.7) to see if a protein could be correlated with the significant increase in succinate uptake seen in cells grown on M9.

The protein profile of LB grown cells differed significantly from that of M9 grown cells. For LB grown cells many proteins are present with major bands at 29K, 43K, 45K, and 70K. Many of these proteins have molecular weights similar to proteins released from M9 grown cells in the presence of low concentrations of Mg^{++} (Fig. 3.6), suggesting that LB grown cells may be more prone to lysis by osmotic shocking. The 52K and 56K proteins seen in the M9 grown cells are not major species in the osmotic shock of LB grown cells. Scanning of the SDS-PAGE gels indicated that the 56K SEP comprised about 20-25% of the total protein released by osmotic shock from M9 grown cells. Glucose, which is known to repress dicarboxylate transport, had no effect on the major protein species in M9 grown cells, although the presence of glucose seemed to increase some of the minor proteins. Other experiments indicated that succinate, NaCl, or buffering with sodium or potassium phosphate, or Tris-HCl had no effect on the protein profiles.

FIGURE 3.7

EFFECT OF GROWTH CONDITIONS ON OSMOTIC SHOCK RELEASABLE PROTEINS

CBT43 grown in LB or M9 (in the presence of either glycerol-succinate or glucose as the carbon source) were subjected to 1 mM EDTA-sucrose followed by 5 mM $MgCl_2$ osmotic shock. The osmotic shock released proteins were run on SDS-PAGE and stained with Coomassie blue. The growth media are indicated at the top. 1-5 designate different stock cultures of CBT43 available in the laboratory. The amount of protein loaded on is equivalent to that released from 2.5 OD_{600} units.


MICROCOPY RESOLUTION TEST CHART
NBS 1010a
(ANSI and ISO TEST CHART No. 2)
1.0
1.1
2.5
2.2
2.0
1.8


LB
M9
GLYCEROL-
SUCCINATE
M9
GLUCOSE
94
67
43
30
20
1 2 3 4 5 1 2 3 4 5 1 2 3 4 5

3.3.7 Optimization of Aspartate-Sepharose Chromatography for Purification of SEP

3.3.7.1 Osmotic Shock From M9 and LB Grown Cells

Osmotic shock fluid from M9 and LB grown cells was passed through aspartate-Sepharose. The pattern of the proteins eluted with succinate is shown in Fig. 3.8. The majority of the osmotic shock releasable proteins were not retained by the column (i.e. Fig. 2.1). In the case of the M9 grown cells, the 56K SEP was retained while the other major species, the 52K protein, was not. The 200 mM succinate eluted material was primarily SEP with residual levels of the 52K protein. The amount of contaminating 52K in this case was higher in LB grown cells, but it is clear that the column was preferentially retaining SEP.

3.3.7.2 Effect of pH

As minor bands were co-purified with the SEP, attempts were made to optimize the elution to remove these contaminants. Changing the pH of the column buffer was shown to have a significant effect on the elution profiles (Fig. 3.9). The SDS-PAGE protein profiles (Fig. 3.10B) indicate that the contaminants did not change dramatically but the amount of SEP was reduced drastically as the pH was increased above 6.6. At pH 8 the eluted material was mostly "contaminants" (i.e. the co-eluting minor proteins). An "early peak" often occurred in the elution profile when the columns were run at pH 6.6. This eluted either late in the wash or soon after the addition of succinate (before a measurable conductivity increase due to succinate). The proteins present in this early peak (see Fig. 3.11) were similar to those in the total osmotic shock, with the majority of the protein being the 52K protein.

FIGURE 3.8

ASPARTATE-SEPHAROSE CHROMATOGRAPHY OF OSMOTIC SHOCK FLUID FROM LB AND M9 GROWN CELLS

Osmotic shock fluid from LB or M9 grown CBT43 was passed through asparate-Sepharose (in 10 mM phosphate, pH 6.6, 5 mM EDTA, 0.002% PMSF) and eluted with 200 mM succinate in the same buffer.

T - Total osmotic shock loaded on

P - Pass through (non-retained proteins)

Eluted - Peak fractions eluted with 200 mM succinate
1 and 2 refer to different columns of the same osmotic shock preparation.

P', 1', and 2' refer to a second M9 preparation

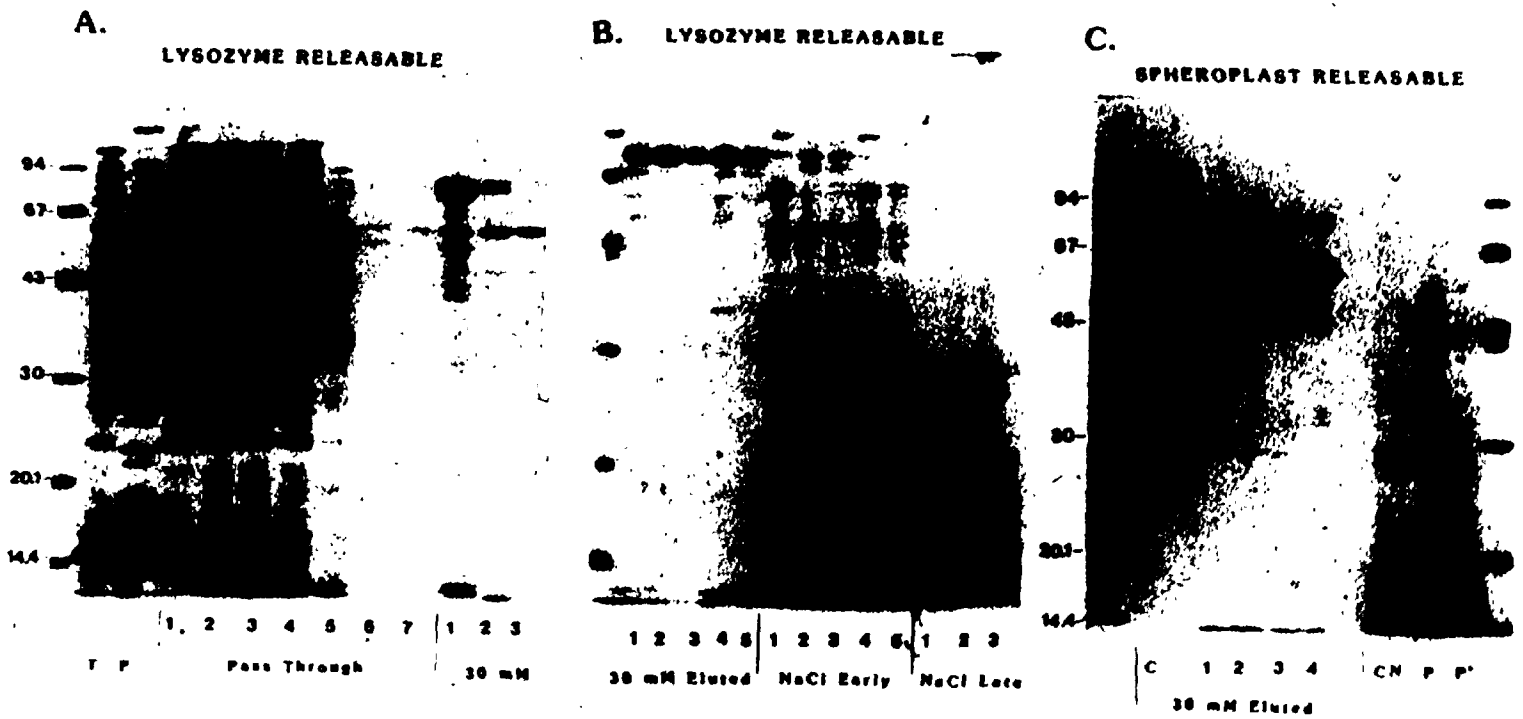

A.
LYSOZYME RELEASABLE
94
67
43
30
20.1
14.4
1 2 3 4 5 6 7 1 2 3
T P Pass Through 30 mM
B. LYSOZYME RELEASABLE
1 2 3 4 5 1 2 3 4 5 1 2 3
30 mM Eluted NaCl Early NaCl Late
C.
SPHEROPLAST RELEASABLE
94
67
43
30
20.1
14.4
C 1 2 3 4 CN P P'
30 mM Eluted

FIGURE 3.9

EFFECT OF pH ON ASPARTATE-SEPHAROSE ELUTION PROFILES OF OSMOTIC SHOCK FLUID

Osmotic shock fluid from M9 grown CBT43 was loaded onto columns of aspartate-Sepharose (17 x 1.5 cm) equilibrated with 10 mM sodium phosphate, 5 mM EDTA at the indicated pH values. After washing with 2 column volumes of buffer, protein was eluted with 200 mM succinate in the same buffer. Protein and succinate in the eluate were monitored. Protein was monitored by its absorbance at 280 nm and succinate was detected by its conductivity. The profiles were recorded from the end of the wash. Slight differences in elution position may reflect differences in column or fraction size (generally about 1.5 ml).

(A) pH 6.6

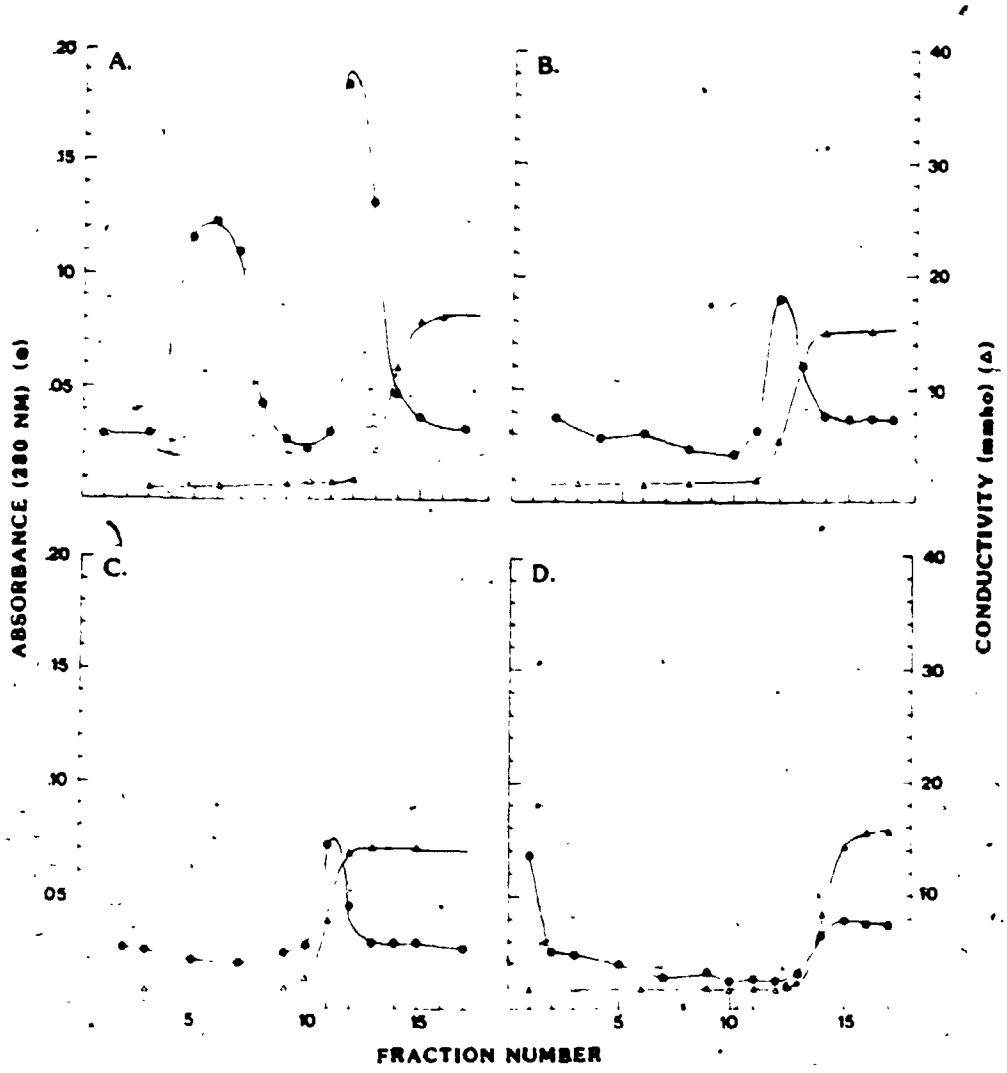
(B) pH 7.0

(C) pH 7.5

(D) pH 8.0

(●) - Absorbance (280 nm)

(△) - Conductivity (mmho)


A.
B.
C.
D.
ABSORBANCE (280 NM) (●)
CONDUCTIVITY (mmho) (△)
FRACTION NUMBER
.20
.15
.10
.05
40
30
20
10
5
10
15

3.3.7.3 Effect of Ionic Strength

The ionic strength of an affinity column buffer can affect protein interaction with the column especially if the interaction is ionic (increasing ionic strength should decrease the interaction) or hydrophobic (increasing ionic strength should increase the interaction). The asparate–Sepharose columns were run in 10 to 100 mM sodium phosphate containing 5 mM EDTA, at pH 6.6 or pH 7.0. It can be seen (Fig. 3.10B) that the amount of SEP eluted from the column was considerably reduced when the ionic strength of the buffer was increased. The level of "contaminants" (except for those between 43K and 56K) was not significantly changed, indicating that these proteins are still retained by the column at higher ionic strengths.

3.3.7.4 Optimization of Elution Conditions

The results presented above indicate that SEP, at pH 6.6, was retained strongly by the column at low ionic strengths (10 mM sodium phosphate, 5 mM EDTA) but was not retained very tightly at 25 mM sodium phosphate, 5 mM EDTA. On the other hand, at pH 7.0 the contaminants bound equally well at either ionic strength. An elution protocol was implemented in which the protein was loaded in 10 mM sodium phosphate, 5 mM EDTA, pH 6.6 and the SEP was eluted with 30 mM sodium phosphate, pH 7.0. Residual protein bound to the column was then removed with 0.5 M NaCl. The SDS–PAGE protein profile from the aspartate–Sepharose columns run under these "optimized" conditions (Fig. 3.11) indicates that the majority of the SEP eluted with 30 mM phosphate. The purity of the eluted SEP was around 95%. The proteins eluted with 0.5 M NaCl were variable from preparation to preparation but protein bands at 64K, 57K, 35.5K, 29.5K, and 26K were most commonly seen.

FIGURE 3.10

OPTIMIZATION OF ASPARTATE-SEPHAROSE BUFFER CONDITIONS
FOR AFFINITY CHROMATOGRAPHY

(A) Effect of phosphate concentration in running buffer on proteins eluted from aspartate-Sepharose.

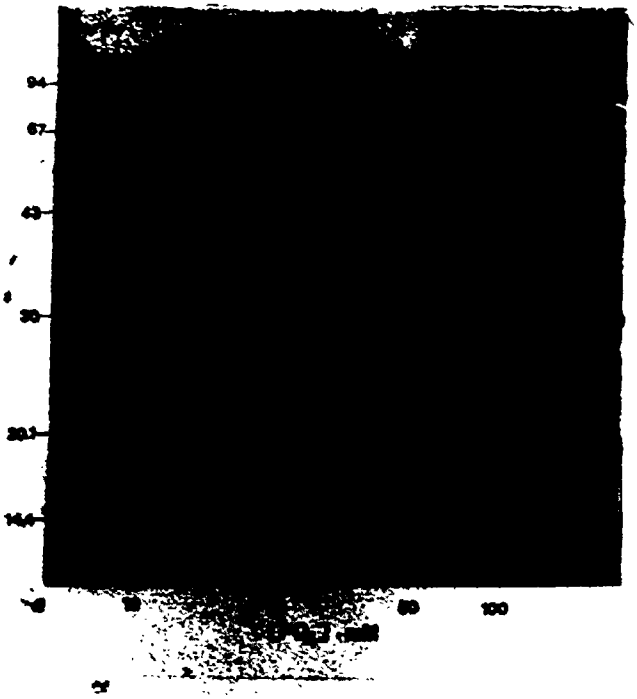
Aspartate-Sepharose columns were run in various concentrations of phosphate buffer (10 to 100 mM, pH 7.0, containing 5 mM EDTA, 0.002% PMSF). Bound protein was eluted with 200 mM succinate. A single peak was observed. SDS-PAGE gels were stained with Coomassie blue. Earliest eluting proteins are at the leftmost side of the respective sections.

(B) Effect of running buffer pH on proteins eluted from aspartate-Sepharose.

Aspartate-Sepharose columns were run at pH's from 6.6 to 8, in 10 mM phosphate, 5 mM EDTA, 0.002% PMSF. Bound protein was eluted with 200 mM succinate. For the pH 6.6 column the sample came from the second peak eluting at the position of succinate (see elution profile Fig. 3.11A)

1 - Just prior to peak
2 - Just after peak
3 - Near end of peak

A. EFFECT OF PHOSPHATE CONCENTRATION



B. pH EFFECT

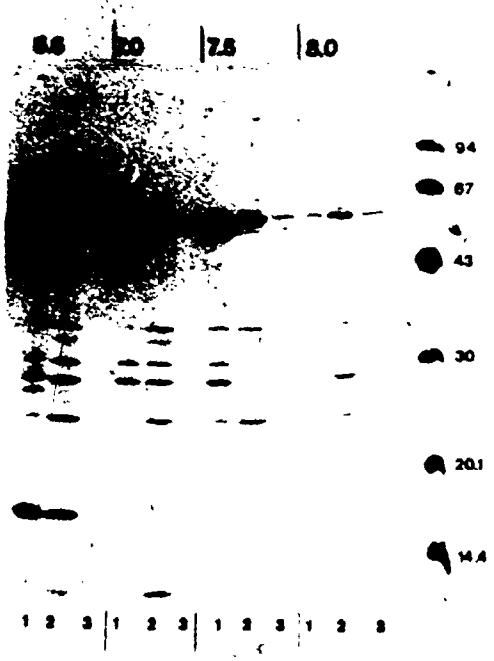


FIGURE 3.11

PROTEIN PROFILES FROM ASPARTATE-SEPHAROSE UNDER OPTIMIZED EXPERIMENTAL CONDITIONS

Osmotic shock releasable proteins were loaded onto aspartate-Sepharose (17 x 1.5 cm) in 10 mM sodium phosphate, pH 6.6; 5 mM K^+-form EDTA; 0.002% PMSF. The column was washed with 2.5 column volumes of buffer and eluted with (1) 30 mM sodium phosphate, pH 7.2 (2) 0.5 M NaCl. The SDS-PAGE gel was stained with Coomassie blue.

The amount of protein loaded onto each lane of the SDS-PAGE gel in the "pass through" samples is equivalent to that released from 3 OD_{600} units of cells. For the 30 mM eluted and late eluting non-bound material, the amount of protein loaded is equivalent to that released from 5 OD_{600} units. For the NaCl eluted material, the amount of protein loaded is equivalent to that released from 100 OD_{600} units.

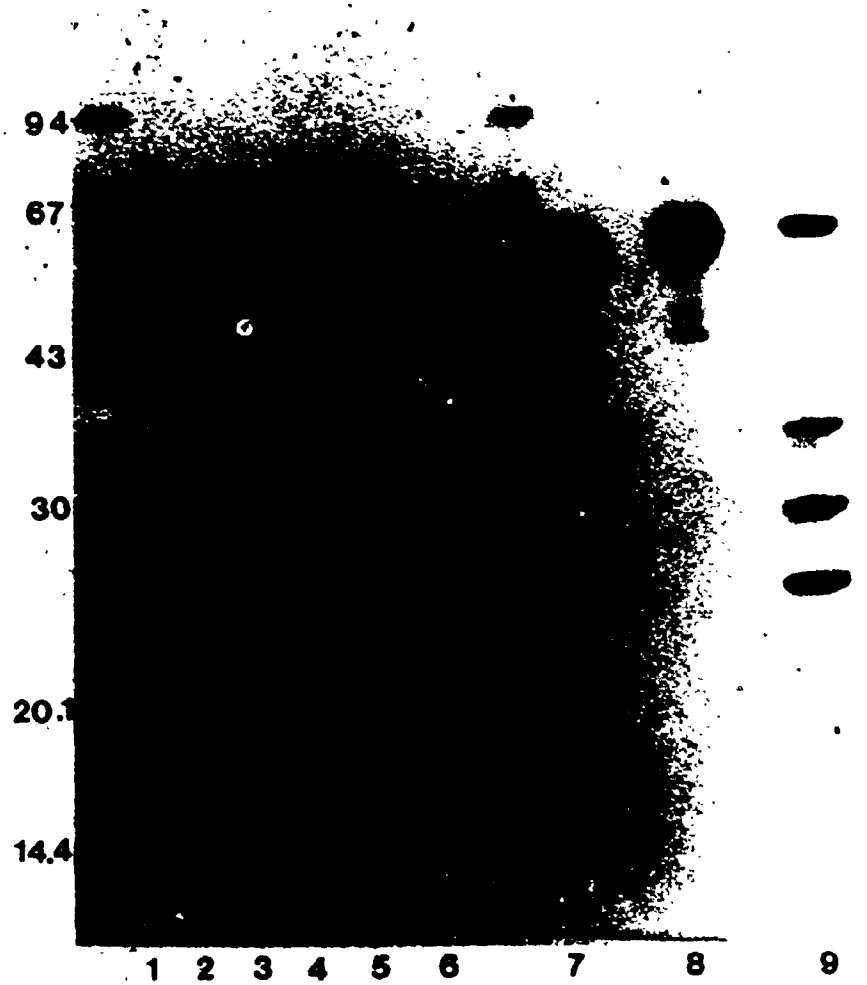
Lane 1-6 - Column pass through.

Lane 7 - Late eluting non-bound material appearing at start of 30 mM elution.

Lane 8 - 30 mM eluted protein.

Lane 9 - 0.5 M NaCl eluted.

OSMOTIC SHOCK RELEASABLE



3.3.8 Aspartate-Sepharose Chromatography of Lysozyme Releasable Proteins

Post-osmotic shock lysozyme releasable proteins were run on aspartate-Sepharose (Fig. 3.12A and B) using the optimized conditions. A protein with the molecular weight of SEP was eluted with 30 mM phosphate, but the amount of this protein was small compared to the osmotic shock releasable sample. A major protein with molecular weight of 80K was also eluted, the nature of which is discussed in the next section. Two protein peaks were eluted with 0.5 M NaCl, the majority of protein was present in an initial "early" peak. The "late" peak was primarily lysozyme which had a strong affinity for the column.

3.3.9 Asparate-Sepharose Chromatography of Proteins Released by Spheroplast Formation

When proteins released by spheroplast formation were separated on aspartate-Sepharose, the major 56K SEP along with an 80K protein were seen (Fig. 3.12C). Lane 1-4 shows the isolation from various isogenic strains. When "pure" lysozyme was run on the aspartate-Sepharose an 80K band was also eluted with 30 mM phosphate (lane C). Thus, the 80K band seen in the lysozyme releasable and spheroplast releasable samples was likely a contaminant of the lysozyme preparation itself and not a population of higher-molecular weight, possibly peptidoglycan associated SEP.

The molecular weight of lysozyme is 14K (74). Analysis of the lysozyme used in these experiments (Fig 3.12C) indicated that there were a considerable number of other proteins present (lane P). A faint 80K band was visible in the original gels. The lower molecular weight material (including the 14K lysozyme) bound very tightly to the aspartate-Sepharose and was only eluted with high NaCl concentrations (lane CN).

FIGURE 3.12

ASPARTATE-SEPHAROSE CHROMATOGRAPHY OF POST-OSMOTIC SHOCK - LYSOZYME RELEASABLE AND SPHEROPLAST RELEASABLE PROTEINS

(A) Post-osmotic shock lysozyme releasable proteins from CBT43.

M9-grown CBT43 that had been osmotically shocked was subjected to a lysozyme treatment to release any "peptidoglycan bound" (46) protein as outlined in "Experimental Procedures". The amount of protein in each lane is equivalent to that released from about 3 OD_{600} units of cells for the "total" and "pass through", and about 10 units of cells for the eluted material. The SDS-PAGE gels were stained with Coomassie blue.

T = Total aspartate-Sepharose load-on.
P = Pre-column run 'pellet' from 40,000 x g spin.
1-7 = Fractions from non-bound pass through; early to late.
30 mM = 30 mM phosphate eluted; 1-3 are various fractions from peak.

(B) Post-osmotic shock lysozyme releasable proteins from various dicarboxylate transport mutants.

The mutants are described in Chapter 4. Following "30 mM" elution, the columns were eluted with 0.5 M NaCl. An early peak was found "NaCl Early", along with a second peak "NaCl Late".

Lane 1 = CBT43; 2 = BL3; 3 = 6-34; 4 = CBT7; 5 = BL25

(C) Spheroplast releasable proteins from various dicarboxylate transport mutants, and pure lysozyme, on aspartate-Sepharose.

Spheroplast releasable proteins (prepared by EDTA-lysozyme treatment of whole cells as described in "Experimental Procedures") as well as pure lysozyme (Sigma) were run on aspartate-Sepharose. The columns were eluted with 30 mM phosphate and 0.5 M NaCl. The amount of protein in each lane of the 30 mM eluted material is equivalent to approximately 3 OD_{600} units of cells.

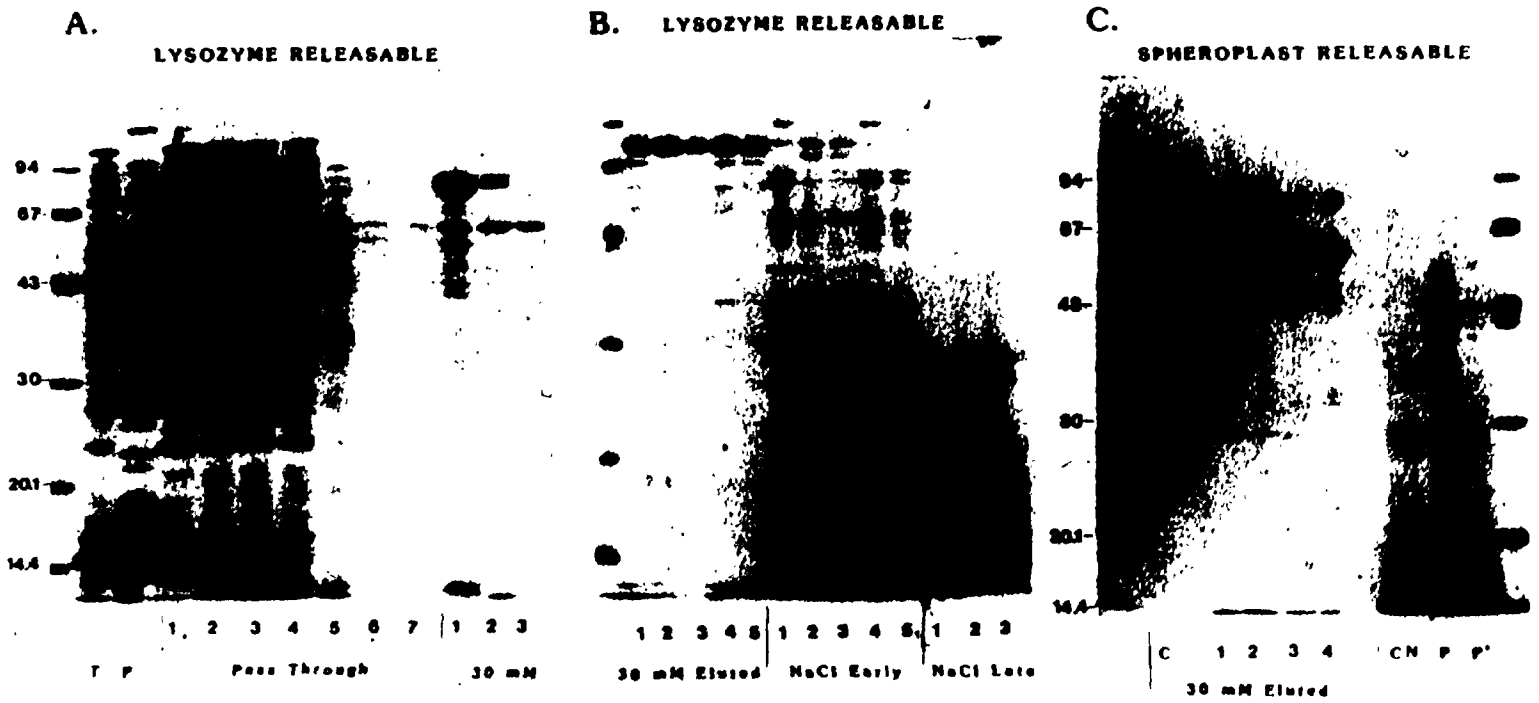
The left side of the gel shows proteins eluted by 30 mM phosphate:

C = from commercial lysozyme preparation
1 = CBT43

Lanes 2-4 are various isogenic Mu-phage insertion transport mutants:

2 = 4-31
3 = 6-34
4 = 6-1

CN = Commercial lysozyme eluted with 0.5 M NaCl
P = "Pure" lysozyme used for spheroplast preparation
P'= A second preparation of lysozyme (also from Sigma)


A.
LYSOZYME RELEASABLE
94
67
43
30
20.1
14.4
1 2 3 4 5 6 7 1 2 3
T P
Pass Through
30 mM
B.
LYSOZYME RELEASABLE
1 2 3 4 5 1 2 3 4 5 1 2 3
30 mM Eluted
NaCl Early
NaCl Late
C.
SPHEROPLAST RELEASABLE
94
67
43
30
20.1
14.4
C 1 2 3 4 CN P P'
30 mM Eluted

3.4 DISCUSSION

To increase the yield of SEP various optimization steps were carried out, as outlined in this chapter. As well as providing increased quantities of pure SEP these studies also provide information on (i) physiological control of the dicarboxylate transport system; (ii) the state of the cell envelope in cells grown under a variety of conditions (as determined by cell fragility); (iii) the localization of SEP; and (iv) a comparison between DBP and SEP as indicated by its affinity for aspartate-Sepharose.

Maximal succinate transport activity was obtained when cells were grown in M9. The activity was at least 50% higher than the maximum level seen in LB grown cells. The phase of growth and aeration of the culture media was important, especially when larger volumes of cells were grown. The kinetic parameters indicated that the increased uptake in M9 grown cells was due to the increased transport capacity of the cells.

A number of proteins, including SEP, were released by EDTA-sucrose treatment. As mentioned in Section 2.1, EDTA-sucrose treatment was shown to release a population (about 10%) of a protein identified as DBP, which by various criteria was assigned a cell surface location (45-47). On the basis of these previous studies it was assumed that the EDTA-sucrose releasable SEP represented a cell surface population as well. A degree of caution must be exercised here, as EDTA is known to release up to 50% of the LPS from cells (73) and Tris and EDTA were shown to cause outer membrane "blebbing" (74). One of the proteins released by EDTA-sucrose (Fig. 3.5) appears to be porin (37K) indicating

outer membrane damage may be occurring. On the other hand, the other major outer membrane protein OmpA (molecular weight = 34K) did not appear to be released indicating that protein release was at least somewhat selective.

SDS-PAGE profiles of osmotic shock fluid from M9 grown CBT43 revealed the presence of 2 major protein bands at 52K and 56K. The two proteins seem to be expressed constitutively in cells grown in minimal media. On the other hand, the osmotic shock from LB grown CBT43 contained a large number of other proteins; SEP and 52K were not as prominent as in the M9 grown cells. Thus the levels of SEP in M9 and LB grown cells correlated well with the succinate transport activities in the cells. Unlike the products of the dct system however, the level of SEP was not repressed by glucose.

The osmotic shocking conditions were optimized to give minimal release of cytoplasmic contents from M9 grown cells. Release of RNA, DNA, and β-galactosidase activity was taken as a measure of cytoplasmic membrane leakage. It was found that 5 mM $MgCl_2$ was optimal for minimizing release of cytoplasmic contents. Since only very small amounts of SEP were released by a subsequent lysozyme treatment SEP seems to be almost completely released by the osmotic shock treatment. The formation of spheroplasts also released a large amount of SEP. The above data demonstrates that this protein is located outside the cytoplasmic membrane in the cell envelope. Operationally at least the majority of SEP appears to be "periplasmic" as evidenced by its release by osmotic shock. It should be noted that it is possible SEP may be tightly bound to the outside of outer membrane; a part of the population is released by EDTA-sucrose while the majority is released only when the

EDTA treated cells are subjected to lower ionic strength, or treated with lysozyme.

Analysis of the aspartate–Sepharose running and elution conditions indicated that optimal binding of SEP to the column occurred at low ionic strengths (10 mM phosphate, 5 mM EDTA) at pH 6.6. SEP was eluted with 30 mM phosphate, pH 7–7.2 indicating that SEP bound to aspartate–Sepharose relatively loosely. The nature of the interaction seems to be ionic, increasing ionic strength decreases the amount of protein bound. The elution behaviour of SEP differs from that originally reported for DBP by Lo and Sanwal (44). In the original isolation procedure 50 mM phosphate and 50 mM arsenate was used. DBP was retained by aspartate–Sepharose under these conditions whereas SEP would not be.

Using the optimized isolation conditions determined in this chapter, SEP of high purity was obtained in much greater amounts than was isolated previously. A major advantage of the elution protocol was that no succinate was present which would have to be removed before subsequent binding assays. The previously used 200 mM succinate elution requires that the levels of succinate be reduced by 2×10^5 to 2×10^6 times (to give a final concentration of 0.1–1 μM) so that binding can be assayed.

CHAPTER 4
DOES SEP CONTAIN A SUCCINATE BINDING SITE?

4.1 INTRODUCTION

The involvement of a protein in a transport process can be assessed in a number of ways. Those components that possess a substrate recognition site can be identified by their ability to bind with a specific substrate. Transport components that do not have a substrate recognition site can only be assayed by reconstitution studies or by genetic analysis.

A number of different binding assays exist to identify proteins with substrate binding sites. One of the most widely used methods is equilibrium dialysis (67). This method has the disadvantage in that the time required for the substrate to reach equilibrium on both sides of the dialysis membrane is long (usually 16–24 hours). If there is protein degradation, little binding will be seen. On the other hand, if there is bacterial contamination, a false-positive result may be observed if the bacteria can take up the ligand. These considerations are of special importance when the binding assay is done at room temperature.

Rapid binding assays are available that are more suitable for use at room temperature for shorter time periods. These involve separation of the free ligand from the bound ligand, by gel filtration (75); nitrocellulose filtration (76); or use of the "Paulus-cell" ultrafiltration apparatus (77). These assays were used to study the binding of succinate to SEP. The gel filtration and nitrocellulose filtration binding assays involve washing of the column or filter to separate protein from free ligand. Dissociation of the substrate from its binding protein could occur during the washing. Preliminary

experiments using these assays gave no clear indication of binding activity, but it was possible that the washing disrupted any binding that was occurring.

The Paulus-cell ultrafiltration assay (77) involves no washing. An ultrafiltration membrane is placed at the bottom of a small pressure cell, ligand and protein are allowed to bind and placed in the cell, and pressure is applied from a gas cylinder. The pressure forces the buffer and free ligand, but not the protein, through the membrane. While the protein concentration increases the ligand concentration remains constant, thus reducing the chance of dissociation. A residual volume of liquid (in the order of 1-3 µl) remains within the filter itself; a blank, without protein, must also be run to determine this volume.

Mutants are commonly used in transport experiments. Protein profiles of transport mutants are examined to see if mutants are missing or altered in a particular protein. Revertants are then studied to see if the protein alteration is also reversed. Several dicarboxylate transport mutants (and revertants) were examined to see whether SEP could be correlated with changes in transport activity.

Guyer et al (71) recently isolated (from E. coli W) a periplasmic oligopeptide binding protein (OBP) which has physicochemical properties similar to those determined for SEP in Chapter 2. These properties included a molecular weight of 60,000 by SDS-PAGE, pI of 5.95, and co-elution with ovalbumin from gel filtration columns (Sephacryl S-200). This protein, was isolated by anion exchange chromatography and shown to bind to a synthetic tripeptide with a K_d of 0.1 µM. To determine if SEP and OBP were indeed related, polyclonal rabbit antiserum against OBP were obtained and used in Western blots against total osmotic shock fluid.

4.2 EXPERIMENTAL PROCEDURES

4.2.1 Bacterial Strains and Growth Conditions

The bacterial strains are listed in Table 4.1. Cells were grown in LB or M9 media, the composition of which is outlined in section 2.2.2.

4.2.2 Preparation of SEP for Binding

The isolation of SEP was by the optimized procedure outlined in section 3.3.7.4. Binding was generally carried out in 10 mM sodium phosphate, pH 6.6–7.0 since SEP had been shown to bind most strongly to the aspartate–Sepharose under these conditions. The 30 mM eluted protein was diluted to 10 mM and the pH adjusted to 6.6. The protein was then concentrated by ultrafiltration on an Amicon YM–10 membrane (10,000 molecular weight cut-off) at 4°C, and washed twice with the buffer used for the binding assay. When possible, binding assays were carried out immediately. If the protein had to be stored overnight, this was done on ice in the cold room, so that freeze-thawing could be avoided. Determination of protein concentration was by the method of Lowry *et al* (80) using bovine serum albumin as the standard.

4.2.3 "Paulus Cell" Ultrafiltration Binding Measurements

4 mm diameter circles were cut from a YM–10 (Amicon Inc.) membrane using a sharpened brass cork borer. These were soaked in water and stored in 10% ethanol at 4°C until used. Before use the membranes were soaked with 2 changes of sterile water.

The plexiglass ultrafiltration cell (Metalloglass) was soaked in detergent overnight before use to remove any grease or protein, and scrupulously dried. The membranes were put in place, the apparatus assembled, and checked for leaks by passing through 50 µl of sterile water. The protein solution containing radioactive succinate was added

TABLE 4.1

BACTERIAL STRAINS

STRAIN DESIGNATION	RELEVANT GENETIC MARKERS[a]	ORIGIN
CBT43	sdh, frd, thi	Laboratory stock
BL3	cbtA, sdh, frd, thi	Laboratory stock Derived from CBT43 (45)
BL25	cbtB, sdh, frd, thi	Laboratory stock Derived from CBT43 (45)
2-24	dct? or cbt?, sdh, frd, thi	Laboratory stock Derived from CBT43 (78)
4-31	dct?, sdh, frd, thi	Laboratory stock Derived from CBT43 (78)
6-1	cbt?, sdh, frd, thi	Laboratory stock Derived from CBT43 (78)
6-34	cbt?, sdh, frd, thi	Laboratory stock Derived from CBT43 (78)

[a] The abbreviations follow the nomenclature of Bachmann (79).

The genetic markers are sdh - succinate dehydrogenase; frd - fumarate reductase; thi - thiamine requirement; cbtA, cbtB - dicarboxylate binding protein; dct - dicarboxylate transport (cytoplasmic membrane components).

and filtered through to apparent dryness using 40 p.s.i. of nitrogen. The bottom of the filters was washed with 5 ml of ethylene glycol to remove the ligand that had passed through. The apparatus was disassembled and the filters placed in 0.5 ml of sterile water for 15 min. Scintillation fluid was added and the filters were counted in a scintillation counter.

4.2.4 Transport Studies and Protein Profiles of Mutants

Succinate uptake studies were carried out as outlined in section 3.2.2. Cells were grown from a 1:10 inoculum in LB (for 3.25 hours) or M9 (for 4.5 hr). Small scale preparation of osmotic shock releasable proteins for running on SDS-PAGE gels was as outlined in section 3.2.3.3. Aspartate-Sepharose chromatography of osmotic shock releasable proteins from the various mutants was performed using the optimized conditions described in section 3.3.7.4.

4.2.5 Western-Blotting Using Anti-OBP Antibody

Lyophilized rabbit anti-OBP antiserum was obtained from Dr. J.V. Staros (Vanderbilt U., Nashville, TN). This antiserum was reconstituted using sterile deionized water.

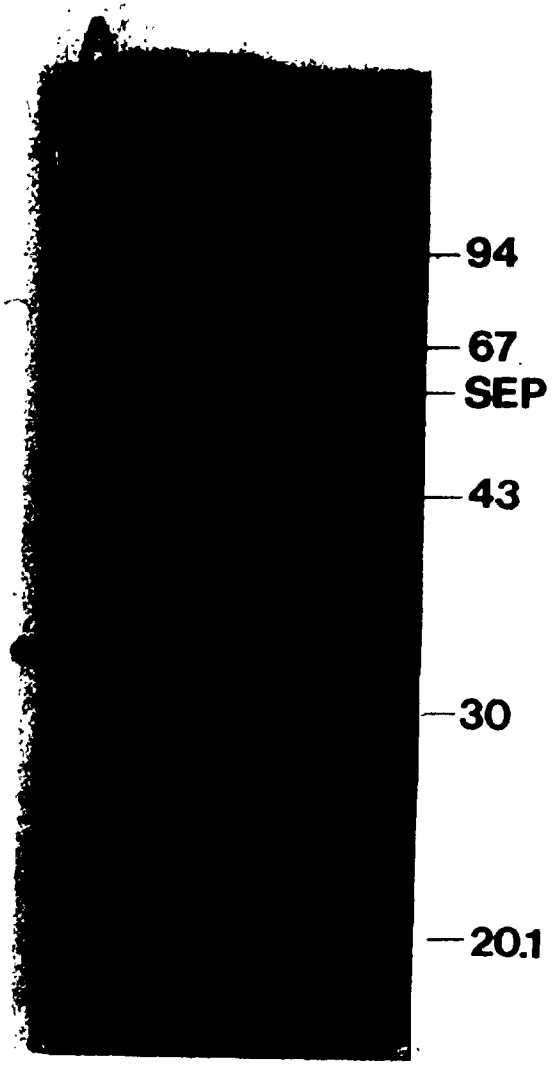
The Western (immuno-)blotting procedure of Towbin *et al* (81) was used to determine antigenic cross-reactivity. Osmotic shock fluid from CBT43 or BL25 was run on SDS-PAGE gels according to Laemmli (59). Proteins were electrophoretically transferred onto nitrocellulose (Schleicher and Schuell, BA83, 0.2 μm) in an E.C. TransBlot apparatus, at 0.5 amps for 90 min, using Tris-glycine Laemmli running buffer containing 20% methanol. The nitrocellulose sheet was stained with 0.05% Amido black to visualize the transferred proteins, destained, and equilibrated with Tris-buffered saline (TBS, 20mM Tris-HCl; 0.85% NaCl;

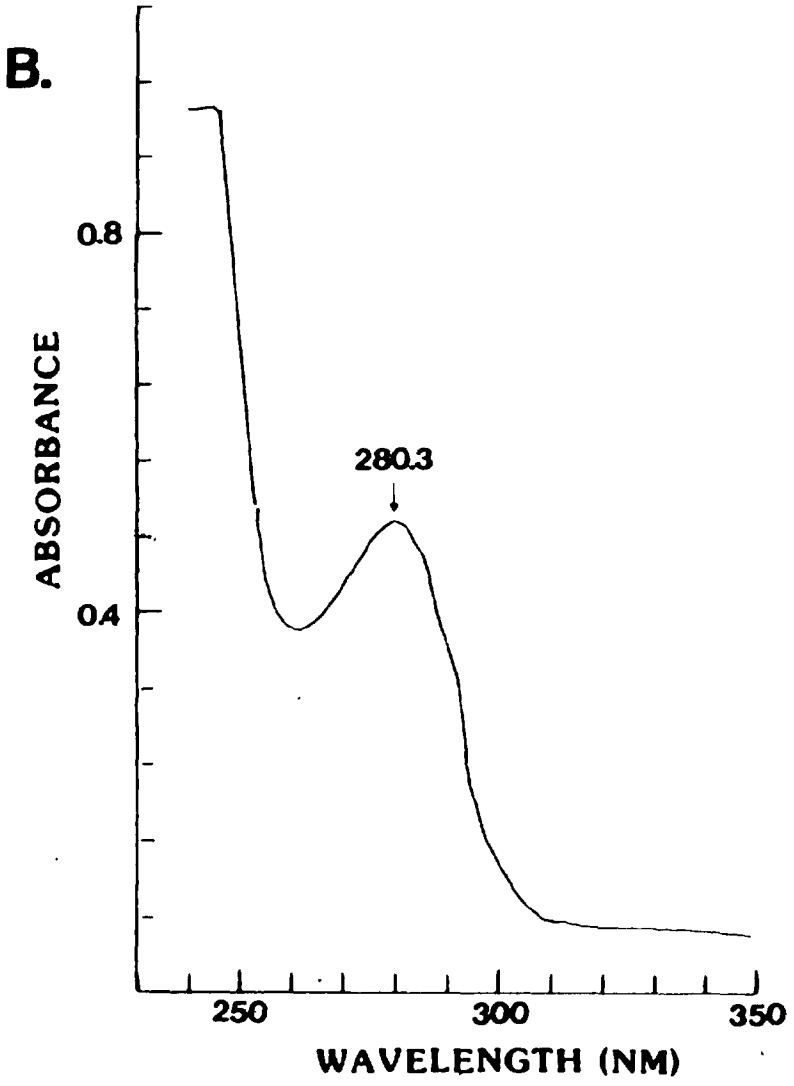
FIGURE 4.1

PURITY OF SEP USED FOR BINDING

(A). Coomassie blue stained SDS-PAGE gel of SEP eluted from aspartate-Sepharose with 30 mM phosphate and equilibrated with 10 mM phosphate, pH 6.6. Molecular weights of standards (Pharmacia) are shown on the right.

(B). U.V. absorbance profile of purified SEP. The λ_{max}, determined automatically, is shown.


94
67
SEP
43
30
20.1


B.
ABSORBANCE
0.8
0.4
280.3
250
300
350
WAVELENGTH (NM)

0.02% NaN_3; pH 7.4). Non-specific protein binding sites were blocked with "Blotto" which consists of 5% Carnation skim milk powder (82) in TBST (TBS containing 0.05% Tween-20 (Sigma)) for 2 hr at room temperature. The antiserum (diluted 1:500 in Blotto) was applied for 1 hour. The blot was washed at least 6 times with TBST, and alkaline phosphatase conjugated goat anti-rabbit IgG (Jackson Laboratories), diluted 1:5000 in TBST, was then applied for 1 hour.

Following washing in TBST, the colour development substrates of Leary et al (83) in alkaline phosphatase buffer (APB) (100 mM Tris, pH 9.0; 100 mM NaCl; 5 mm $MgCl_2$) were added as outlined in the Promega Biotec Protoblot handbook. The coloured substrates were 33 µl of Nitro blue tetrazolium (Sigma) (50 mg/ml in 70% dimethyl formamide) and 16.5 µl of 5-bromo-4-chloro-3-indolyl phosphate (p-toluidine salt) (BCIP, Sigma) (50 mg/ml in DMF) per 5 ml of APB. After colour development the nitrocellulose was washed with 10 mM EDTA, rinsed in deionized water, and stored dry.

4.3 RESULTS

4.3.1 "Paulus-Cell" Ultrafiltration Binding Assays

The Paulus-cell ultrafiltration assay (77) was utilized because it involves no washing. The purity of the protein used for binding is shown in Fig. 4.1. Fig. 4.1A indicates that the protein is essentially free of contaminating proteins. Fig 4.1B indicates that the SEP is free of DKF (which has absorbance maximum of 257 nm). Results of the binding assay are shown in Tables 4.2 to 4.4.

The amount of liquid retained by the filter was on the order of 1.5 to 2 µl (containing 36-48 pmoles of succinate). It can be seen that

TABLE 4.2

PAULUS-CELL ULTRAFILTRATION BINDING ASSAYS WITH VARIOUS FRACTIONS

ORACTION	VOLUME FILTERED (µl)	PROTEIN CONCENTRATION (mg/ml)	PROTEIN ADDED (µg)	PROTEIN ADDED (pmoles)	SUCCINATE RETAINED (pmoles)
Blank	150				38 ± 2
A: CBT43, Fractions from Aspartate-Sepharose					
Pass-Through	100	2	200		36 ± 4
	300	"	600		38 ± 3
SEP	150	0.3	45	800	42 ± 6
	450	"	135	2400	40 ± .5
"in Tris"	150	"	45	800	39 ± 4
	450	"	135	2400	47 ± 1
0.5 M NaCl Eluted	90	0.1	9		33 ± 4
	150	"	15		31 ± 4
	450	"	45		42 ± 4
	600	"	60		46 ± 4
B: BL25, Fractions from Aspartate-Sepharose					
Pass-Through	150	0.45	68		45 ± 4
	225	"	100		46 ± 2
	450	"	200		53 ± 4
SEP	150	0.25	38	680	53 ± 4
	450	"	115	2000	56 ± 2
"in Tris"	150	"	38	680	50 ± 5
	450	"	115	2000	47 ± 4
0.5 M NaCl Eluted	150	0.085	13		37 ± .2
	450	"	38		46 ± 2

Binding of [^{14}C]-succinate to various osmotic shock fractions separated by aspartate-Sepharose affinity chromatography. Binding using the Paulus-cell was carried out as outlined in "Experimental Procedures". The binding reaction contained protein at the indicated concentrations, and carrier-free succinate (1000 dpm/9.45 pmoles), at a concentration of 24 µM (i.e. 24 pmoles/µl). The "blank" contained no protein, and the succinate retained is indicative of trapping by the filter. The amount trapped (38 pmoles) is equivalent to about 1.6 ul. Binding was carried out for 1 hour in a microfuge tube and the protein solution was centrifuged for 5 min immediately prior to addition to the ultrafiltration well. Isolated proteins were dialysed overnight against 10 mM potassium phosphate, pH 6.6 except for "in Tris" which was dialysed against 20 mM Tris-HCl, pH 7.2.

TABLE 4.3
EFFECT OF DIALYSIS ON SUCCINATE BINDING ACTIVITY

FRACTION	VOLUME FILTERED (µl)	PROTEIN CONCENTRATION (mg/ml)	PROTEIN ADDED (µg)	PROTEIN ADDED (pmoles)	SUCCINATE RETAINED (pmoles)
A: FRESHLY ELUTED:					
EDTA-Sucrose SEP	400	0.015	6	107	44 ± 4
0.5 M NaCl Eluted	350	0.024	8		48 ± 11
Osmotic Shock SEP					
"Early"	200	0.29	58	1040	46 ± 4
"Mid"	200	0.32	64	1140	40 ± 2
"Late"	200	0.16	32	570	40 ± 4
0.5 M NaCl Eluted	350	0.064	22		48 ± 5
B: DIALYSED:					
EDTA-Sucrose SEP	150	0.04	6	107	37 ± 5
	450	"	18	320	41 ± 2
0.5 M NaCl Eluted	150	0.05	8		35 ± 4
	450	"	23		47 ± 3
Osmotic Shock SEP					
"Early"	150	0.25	38	670	35 ± 3
	450	"	113	2000	46 ± 1
"Mid"	150	0.28	42	750	34 ± .3
	450	"	126	2250	42 ± 2
"Late"	150	0.13	20	348	37 ± 3
	450	"	60	1045	45 ± 12
0.5 M NaCl Eluted	150	0.16	24		43 ± .3
	450	"	72		56 ± 6

Paulus-cell binding assays were carried out as outlined in Table 4.2. Proteins released from cells by EDTA-sucrose or osmotic shocking were loaded onto aspartate-Sepharose columns without freezing. Protein in the binding studies was either used directly from the column or dialysed overnight against 10 mM potassium phosphate (pH 6.6) and the binding repeated. Protein isolated from EDTA-sucrose releasable material was concentrated on an Amicon YM10 membrane after dialysis.

TABLE 4.4

EFFECT OF PMSF ON SUCCINATE BINDING ACTIVITY

FRACTION	VOLUME FILTERED (µl)	PROTEIN CONCENTRATION (mg/ml)	PROTEIN ADDED (µg)	PROTEIN ADDED (pmoles)	SUCCINATE RETAINED (pmoles)
BLANK	150	0	0	0	32 ± 2
	450				41 ± 3
Pass-Through					
– PMSF	450	N.D.			40 ± 4
+ PMSF	450				44 ± 5
SEP					
– PMSF	450	0.25	115	2000	44 ± 5
+ PMSF	450	0.25	115	2000	40 ± 13
0.5 M NaCl					
– PMSF	150	0.22	33		34 ± .2
	450		99		38 ± .7
+ PMSF	150	0.163	24		36 ± 1.4
	450		73		42 ± 2

N.D. = Not Determined

Succinate binding using the Paulus-cell ultrafiltration assay was carried out as described in Table 4.2. Aspartate-Sepharose columns of osmotic shock releasable proteins were run in the presence ("+ PMSF") or absence ("– PMSF") of 0.002% PMSF. Isolated fractions were dialysed against 10 mM sodium phosphate, pH 6.6 (with or without 0.002% PMSF) prior to binding. The NaCl eluted fraction was concentrated by ultrafiltration.

none of the binding assays gave results that were very much different than this. All binding assays were carried out after a pre-centrifugation (5 min at 11,000 x g). Various fractions were utilized including EDTA-sucrose and osmotic shock aspartate-Sepharose "pass-through", SEP, or 0.5 M NaCl eluted. Binding was assayed in the presence of phosphate or Tris, with freshly eluted protein or after overnight dialysis, but in no case was any significant binding seen.

It was felt that the presence of PMSF in the elution protocol, added to prevent proteolysis, may have been in fact affecting the binding protein itself. However, when a comparison was made between protein isolated and dialysed in the presence or absence of PMSF, no difference was seen (Table 4.4).

4.3.2 Studies with mutants

A number of dicarboxylate transport mutants were available in the laboratory. These included BL25 and BL3, described by Bewick and Lo (45) as having mutations in DBP. They are isogenic with the "wild-type" CBT43. The mutation in BL25 was thought to be due to a reduction in all forms of DBP (the *cbtB* mutant) and in the case of BL3 the mutation was thought to occur in the cell surface "transport component recognition site" (*cbtA* mutation) (45). BL3 and BL25 exhibited slight growth on minimal malate plates after 48-72 hours. The colonies exhibited a "spotty" phenotype characterized by a region of poor growth with areas of more intense growth. Spontaneous revertants of BL25 and BL3 were selected by picking the largest "spots", restreaking on minimal malate plates, and repeating.

From the SDS-PAGE profiles of osmotic shock released proteins (Figure 4.2) it can be seen that the major 52K protein band is missing

FIGURE 4.2

SDS–PAGE PROFILES OF OSMOTIC SHOCK RELEASABLE PROTEINS FROM DICARBOXYLATE TRANSPORT MUTANTS GROWN IN VARIOUS MEDIA

Small scale cultures were subjected to EDTA-sucrose and 5 mM $MgCl_2$ - osmotic shock treatment and the proteins prepared for SDS–PAGE gel electrophoresis as outlined in Section 3.2.3.3. Equivalent amounts of osmotic shock releasable proteins (about 30 ug) were added to each lane.

(A) - Cells grown in LB

(B) - Cells grown in M9 supplemented with glycerol and succinate

(C) - Cells grown in M9 supplemented with glycerol–succinate or glucose

LANE:

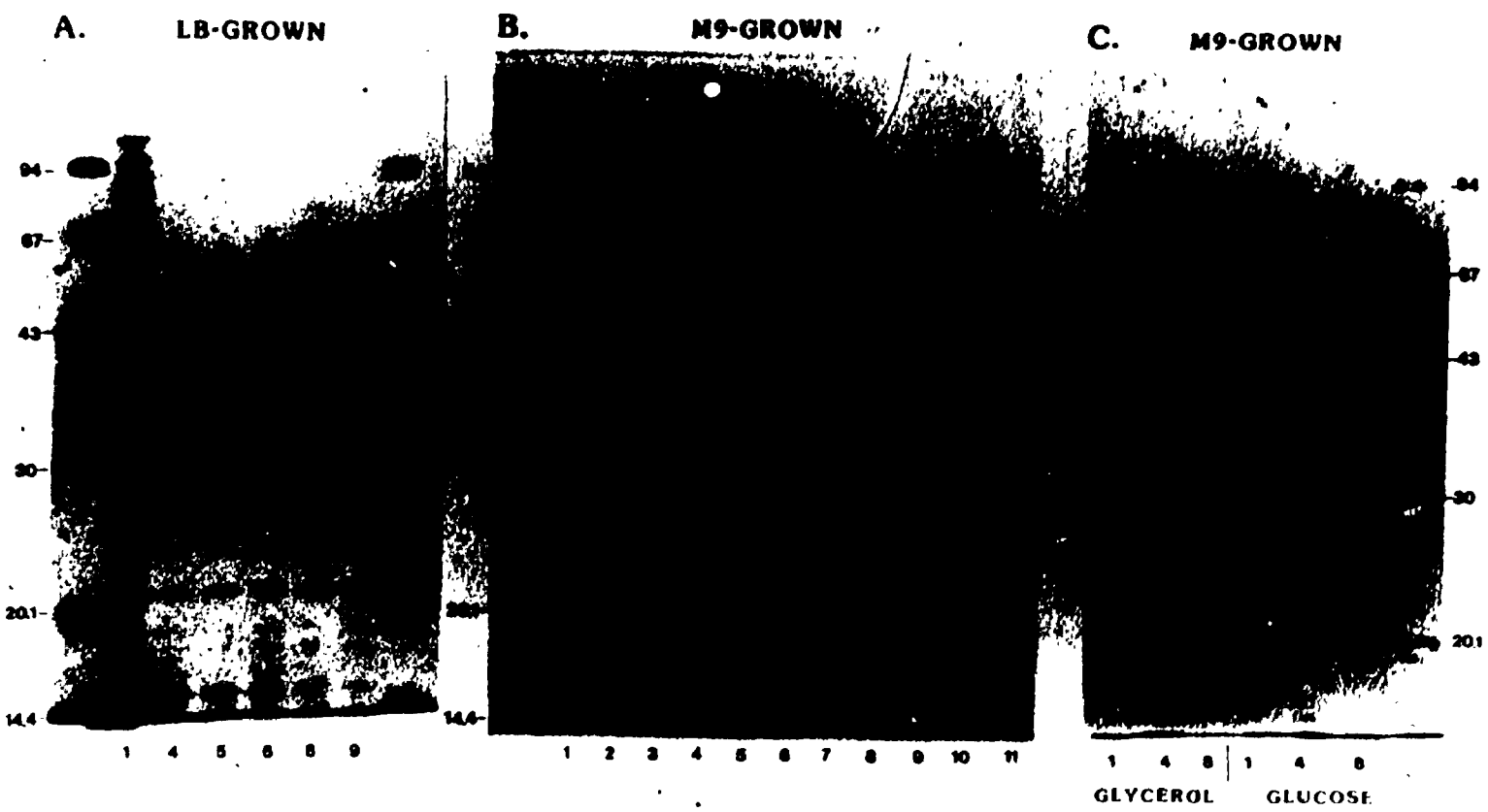
1–3 - CBT43

4 - BL25

5,6,7 - BL25 Revertants

8 - BL3

9,10,11 - BL3 Revertants


A.
LB-GROWN
B.
M9-GROWN
C.
M9-GROWN
94
67
43
30
20.1
14.4
1 4 5 6 8 9
1 2 3 4 5 6 7 8 9 10 11
1 4 8 1 4 8
GLYCEROL
GLUCOSE

in BL25 (lane 4), grown in LB or M9. However, BL25 revertants (lanes 5,6,7) which had uptake ability at least as great as CBT43 in LB, were still missing this protein. Thus, this 52K protein does not seem to be important for succinate uptake. It was still present in BL3 (lane 8) and its revertants (lanes 9-11).

Mu-phage insertion succinate transport mutants of CBT43 were isolated by K. Walker (78) based on their ability to grow on the toxic analogues β-chloro-lactate and difluorosuccinate. Cells able to grow on difluorosuccinate, a toxic succinate analogue, are impaired in their ability to transport dicarboxylic acids. β-Chlorolactate is a toxic analogue of lactate. Previous studies have shown that (i) the dicarboxylate binding protein was able to bind lactate (44) and (ii) β-chlorolactate resistant bacteria had dicarboxylate transport mutations at the level of the periplasmic protein (46).

Growth of Mu-phage insertion mutants on minimal malate plates provided initial indications of differences in dicarboxylate transport ability. Some strains exhibited no growth on malate plates (e.g. strains 4-31 and 2-24). Other strains showed "spotty" growth after 48 hrs (e.g. strains 6-1, 6-34), a result similar to BL25 and BL3. It was felt that the mutation in this case may be at the level of the periplasmic binding protein. The slight growth and uptake may be due to diffusion across the periplasmic space to the intact cytoplasmic membrane transport components. Tight mutations may be due to mutations in the cytoplasmic membrane proteins.

The succinate transport activity of LB grown and M9 grown Mu-insertion mutants was measured. For strains 6-1 and 6-34 the activity is intermediate and succinate transport increase relative to CBT43 in

FIGURE 4.3

SUCCINATE UPTAKE WITH MU-PHAGE INSERTION MUTANTS

CBT43 and Mu-phage insertion mutants grown in LB or M9 were assayed for succinate uptake ability (in 2 uM succinate). Panel A shows uptake for LB grown cells and panel B shows uptake for M9 grown cells.

(●) - CBT43

(▲) - 2-24

(○) - 4-31

(□) - 6-34

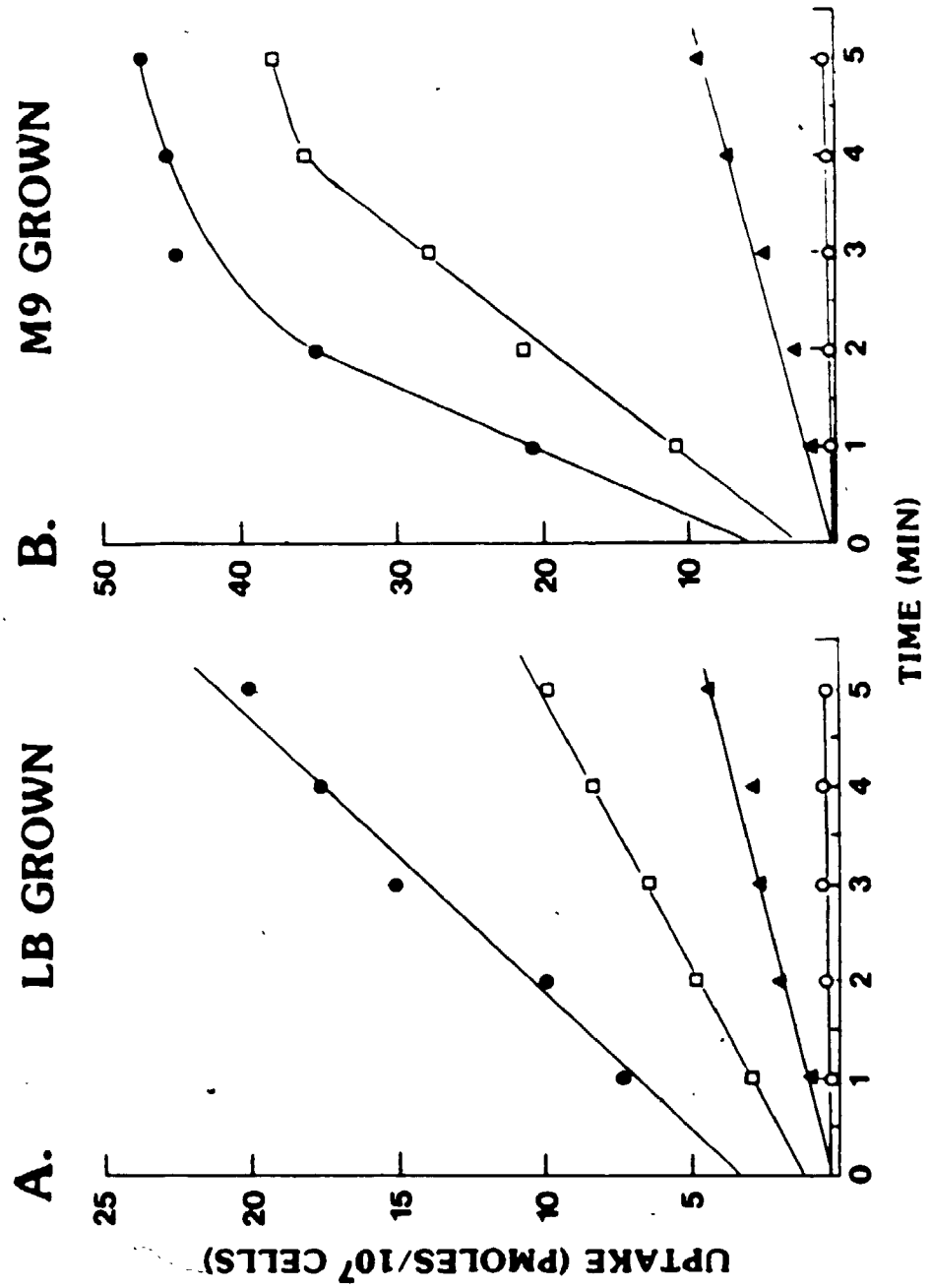

A.
LB GROWN
B.
M9 GROWN
UPTAKE (PMOLES/10⁷ CELLS)
TIME (MIN)
0
5
10
15
20
25
10
20
30
40
50
1
2
3
4
5

FIGURE 4.4

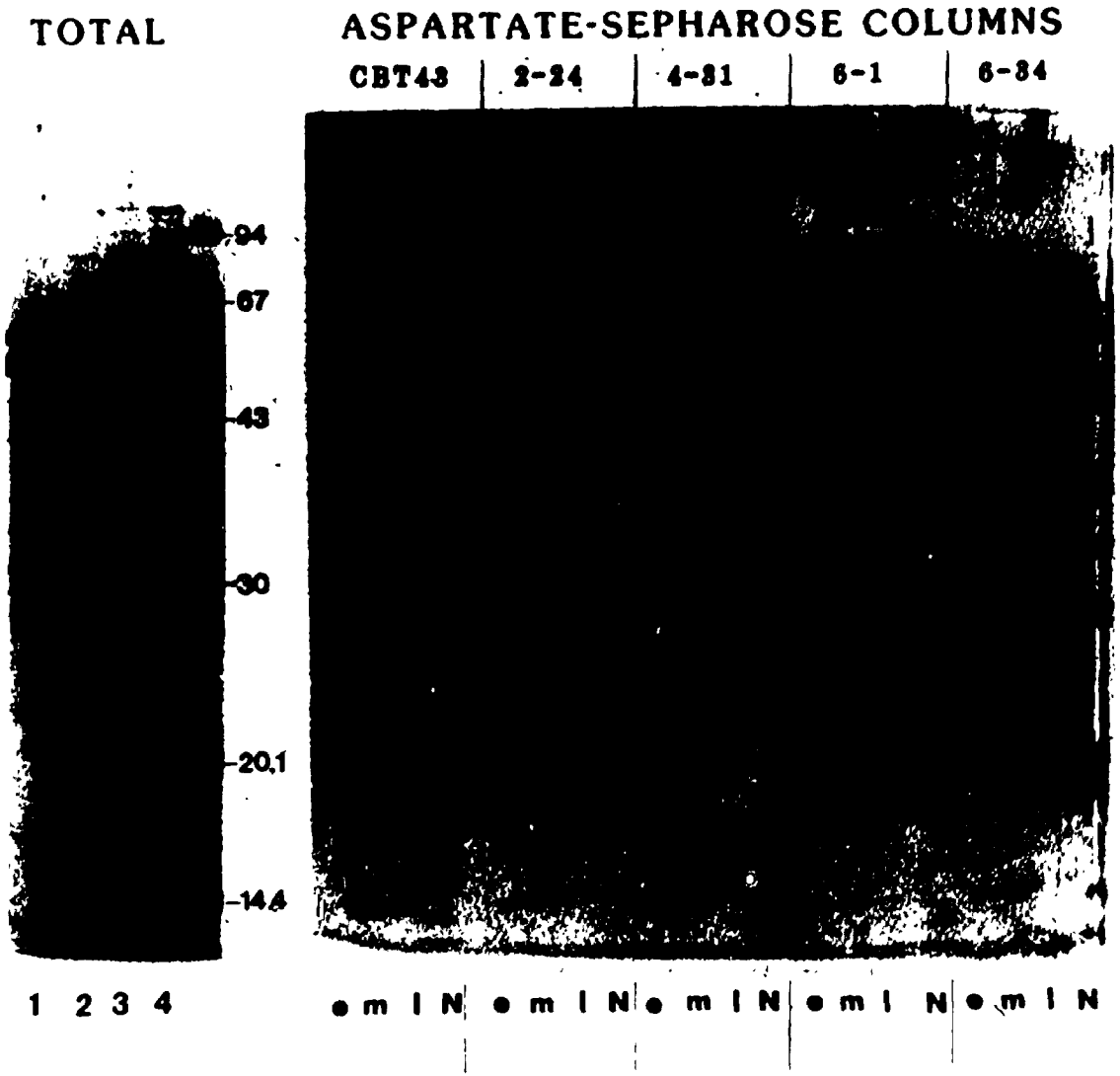
ASPARTATE-SEPHAROSE CHROMATOGRAPHY OF OSMOTIC SHOCK RELEASABLE PROTEINS FROM VARIOUS MU-PHAGE INSERTION MUTANTS

The left panel represents total osmotic shock releasable proteins from M9-grown Mu-phage insertion mutants of CBT43 with reduced succinate transport. Succinate transport activity was shown in Fig. 4.6.

Lane 1 = 2-24; 2 = 4-32; 3 = 6-1; 4 = 6-34

The right panel represents proteins eluted from aspartate-Sepharose by 30 mM phosphate and 0.5 M NaCl.

e = 30 mM - "early" part of peak
m = 30 mM - "middle" part of peak
l = 30 mM - "late" part of peak
N = 0.5 M NaCl eluted


TOTAL
ASPARTATE-SEPHAROSE COLUMNS
CBT43
2-24
4-81
6-1
6-84
94
67
43
30
20,1
14,4
1 2 3 4
e m I N e m I N e m I N e m I N e m I N

M9. (The uptake profile of 6-34 is shown in Fig. 4.3). Strains 2-24 and 4-31 have lower transport activity (4-31 has virtually none) and levels in M9 do not change relative to CBT43 (Fig. 4.3). Osmotic shock releasable proteins were run on aspartate-Sepharose. No difference can be seen in the proteins eluted (Fig. 4.4) with 30 mM phosphate or 0.5 M NaCl.

Therefore, for all the mutants tested, which exhibited changes in uptake profiles, no change in SEP could be correlated with the change in uptake.

4.3.3 Western Blots Using Anti-Oligopeptide Binding Protein

As the OBP and SEP had similar physicochemical properties, we obtained anti-OBP antiserum prepared by Guyer, to determine immunological cross-reactivity between the proteins. Total osmotic shock releasable proteins from CBT43 and BL25 grown in LB or M9 were probed with the anti-OBP antiserum on Western blots (Fig. 4.7). It is obvious that the 56K SEP is the major protein recognized by the antiserum. The major 52K protein absent in BL25 is not recognized, and shows up as an "empty space" in the CBT43 lane.

4.4 DISCUSSION

Evidence presented in the previous chapters indicate that SEP is not only able to stimulate succinate transport in whole cells but its levels in both M9 and LB grown cells also correlate well with their succinate transport activity. This suggests that SEP may play a role in the transport process. Proteins involved in a transport process may or may not possess a binding site for the substrate and it was the purpose of this work to determine whether such a binding site is present on purified SEP.

FIGURE 4.5

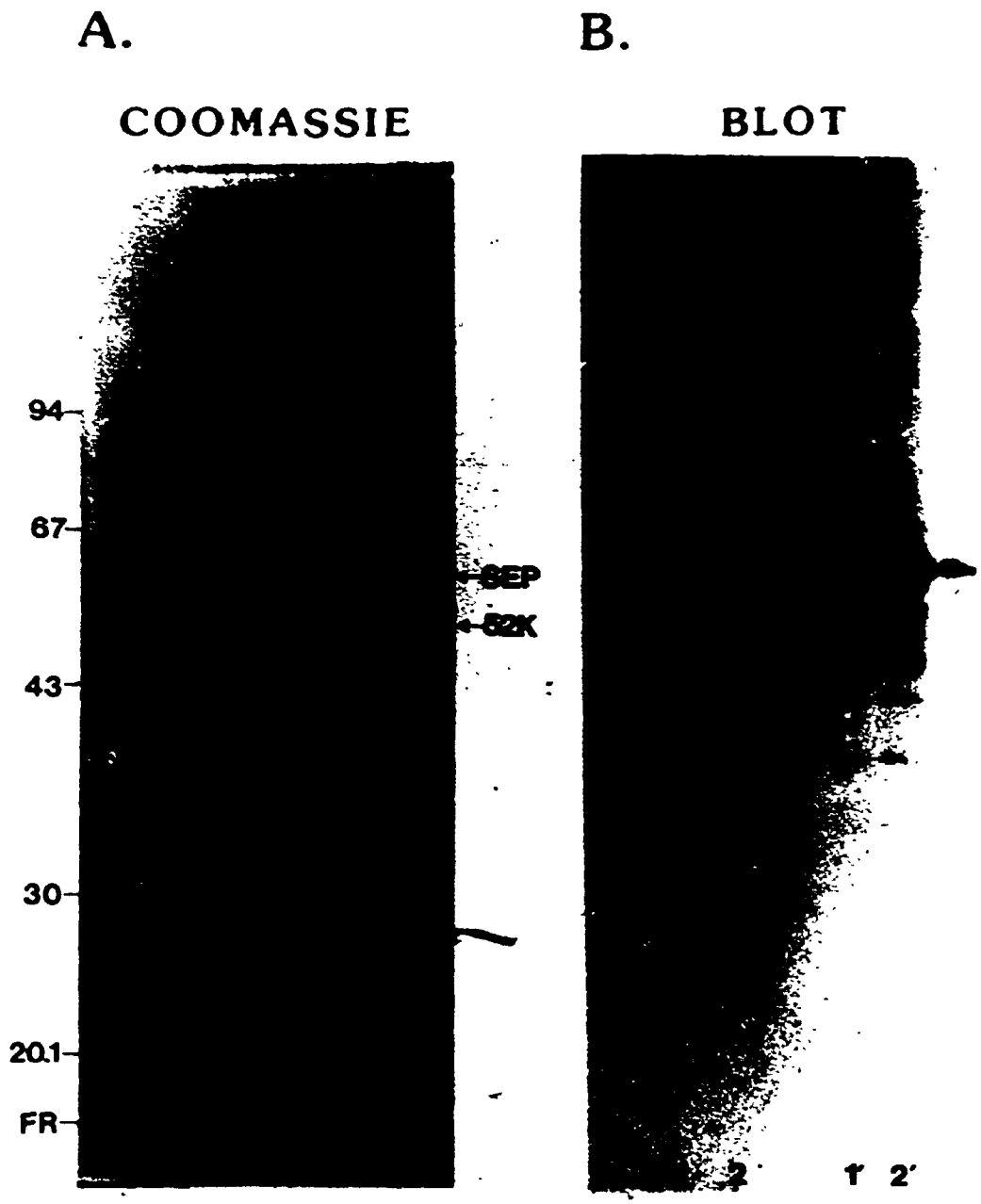
WESTERN BLOTTING OF OSMOTIC SHOCK FLUID WITH
ANTI-OLIGOPEPTIDE BINDING PROTEIN

(A) Coomassie blue stained total osmotic shock.

(B) Western blot using anti-oligopeptide binding protein

Osmotic shock releasable proteins were separated by SDS-PAGE, blotted onto nitrocellulose and probed with anti-oligopeptide binding protein as outlined in "Experimental Procedures". Bound antibody was visualized by alkaline phosphatase conjugated goat anti-rabbit IgG and a colour reaction with NBT/BCIP as described.

Lane 1 = CBT43 - LB-grown
Lane 2 = BL25 - LB-grown
Lane 1' = CBT43 - M9-grown
Lane 2' = BL25 - M9-grown


A.
B.
COOMASSIE
BLOT
94
67
43
30
20.1
FR
SEP
62K
1
2
1'
2'

Preliminary studies using a gel filtration binding assay and the nitrocellulose binding assay to demonstrate succinate binding to SEP were unsuccessful. These negative findings might have been due to a low affinity of the protein for succinate and the possibility that bound ligand was removed by the washing. The K_D reported previously for the dicarboxylate binding protein was around 40 μM (44), considerably higher than other periplasmic binding proteins which were assayed by these methods (generally in the range of 0.1-1 μM (84)).

To eliminate the need for a washing step, the "Paulus-cell" ultrafiltration assay (77) was utilized. This method should be useful in cases where substrate binding affinity is low and bound substrate is rapidly released from the protein. Still, we were unable to detect binding activity in either total osmotic shock, the 30 mM eluted fraction (SEP), or 0.5 M NaCl eluted fraction. The concentration of binding protein may have been too low in the other fractions to measure but the 30 mM eluted fraction contained almost pure SEP at fairly high concentration.

It was felt that the presence of PMSF in the elution protocol, may have been in fact affecting the binding activity. PMSF acts by covalently modifying proteases at the active site serine residue (85). If a similar site is located in or around the active site of the binding protein it is entirely possible that binding activity could be affected. However, no binding was seen if SEP was prepared in the absence of PMSF.

Although quite a number of dicarboxylate transport mutants have been analysed none appeared to be altered in SEP. The major 52K protein

was absent in BL25 but it could not be correlated with changes in transport activity as revertants were still missing this protein.

While characterizing SEP, a report was published (by Guyer et al. (71)) on the properties of the oligopeptide binding protein (OBP) from E. coli W. This protein had physicochemical properties similar to those of SEP. The OBP bound to chemically synthesized oligopeptide substrates with a K_D of 0.1 μM. The molecular weight reported for OBP (around 60K) differed from that of a protein implicated in other studies which had a molecular weight of 52K (86). No purification of, or binding of oligopeptides to, the 52K protein had been reported however.

In order to test the relationship between OBP, SEP, and the 52K protein, antiserum to OBP was obtained. The antiserum was used to probe Western blots of total osmotic shock obtained from LB or M9 grown CBT43 or BL25. A protein at 56K was the major species recognized by the antiserum. The 52K protein was not recognized. Thus, the major protein that was recognized by the anti-OBP corresponds to SEP.

The question that arises from this study is whether the SEP isolated from E. coli K12 strains is identical or related to the OBP isolated by a totally different procedure from E. coli W strains. Genetic analysis of a number of E. coli operons coding for transport systems indicates that they are organized in an almost identical fashion indicating a common ancestry (84). There is also increasing evidence that bacterial transport components from different transport systems possess a sequence of homology (23); and such sequence of homology is also detected in a glycoprotein found in multidrug resistant mammalian cells (87-89). Thus it is not surprising that antibodies directed against the transport components for one transport system may cross-

react with components for another system. Since the synthetic substrate used for assessing oligopeptide binding activity, and purified OBP are not available for our use, the presently available data do not allow us to further determine the relationship between these two proteins.

It is possible that the 16K DBP is a proteolytic product of the 56K SEP, and succinate binding activity of SEP will not be observed *in vitro* unless this modification takes place. Perhaps the defect in some of the *cbt* mutants is at the level of this processing. In BL25 the absence of the 52K protein may reflect the processing defect or the 52K protein may itself be the "processing enzyme". Although no 16K protein cross-reacted with the anti-oligopeptide antibodies which recognized SEP it is possible that the major antigenic sites of the "precursor" protein are not present in the "processed" protein.

The results in this chapter indicate that SEP does not have detectable succinate binding activity. Because of the large amounts of SEP used (i.e. up to 2400 pmoles of pure protein) and because washing was not involved, succinate binding activity should have been detected if a binding site is present. Even if only 10% of the protein retained binding activity there should have been at least 240 pmoles of succinate associated with the filters. But as seen here, variation from background was at most 10 pmoles. The lack of measurable binding activity indicates that pure SEP, by itself, does not bind succinate. Thus the ability of exogenous SEP to stimulate succinate uptake cannot be attributed to an increased number of cell surface substrate binding sites.

CHAPTER 5

INTERACTION OF SEP WITH WHOLE CELLS

5.1 INTRODUCTION

Transport of succinate across the outer membrane of Escherichia coli at low concentrations of substrate, is rate limiting and is dependent on the presence of porins (47).

A population of SEP is releasable from the cell envelope by EDTA-sucrose treatment suggesting a cell surface location. Although SEP exhibits no succinate binding activity, the ability of SEP to increase succinate transport when added to whole cells suggests it may play a role in dicarboxylate transport across the outer membrane. Experiments were therefore undertaken to investigate the interaction between SEP and the cell surface in intact cells. Radioactive SEP, purified by aspartate–Sepharose chromatography, was added to whole cells. The interaction was examined in wild type cells, as well as in previously characterized mutants lacking either one or both major species of porin, in OmpA mutants, in lipopolysaccharide mutants, and in dicarboxylate transport mutants.

5.2 EXPERIMENTAL PROCEDURES

5.2.1 Bacterial Strains

The strains used in these experiments are listed in Table 5.1. The galE mutants are defective in uridine diphosphate galactose epimerase and are unable to form UDP–galactose from UDP–glucose. The LPS of galE strains contains a very incomplete (R_c) core structure containing only three ketodeoxygluconate (KDO), two heptose, and one glucose residue (4). The properties of the outer membrane of these mutants are altered (91).

TABLE 5.1

BACTERIAL STRAINS

STRAIN DESIGNATION	RELEVANT GENETIC MARKERS[a]	ORIGIN
CBT43	*sdh, frd, thi*	Laboratory stock
BL3	*cbtA, sdh, frd, thi*	Laboratory stock Derived from CBT43 (45)
BL25	*cbtB, sdh, frd, thi*	Laboratory stock Derived from CBT43 (45)
LL3	F' *sdh*$^+$, *gal*$^+$/ F$^-$ *sdh, frd, dctA, thi*	Laboratory stock (78)
LL5	F' *argG*$^+$/F$^-$ *argG, dctB thi, (metB, his, leu)*	Laboratory stock (78)
VR42	*aroB, cir, feuB, thi*	Coulton and Braun (89)
PL6	*ompA, aroB, cir, feuB, thi*	Derived from VR42 (89)
JW031	*ompC, ompF, aroB, cir, feuB, thi*	Derived from VR42 (89)
JF568	*pro, aroA, his, purE, ilv, met*	*E. coli* Genetic Stock Centre
JF694	*ompC, ompF, pro, aroA, his, purE, ilv, met*	Derived from JF568 (90)
JF701	*ompC, pro, aroA, his, purE, ilv, met*	Derived from JF568 (90)
JF703	*ompF, pro, aroA, his, purE, ilv, met*	Derived from JF568 (90)
SK11	*galE, asd, leu, pps, thi*	S. Koval (London, Ont.)
SK11R	*asd, leu, pps, thi*	Derived from SK11
4498	*galE, relA, thi*	*E. coli* Genetic Stock Centre

[a] The abbreviations follow the nomenclature of Bachmann (79).

The important genetic markers include: *sdh* - succinate dehydrogenase; *frd* - fumarate reductase; *thi* - thiamine requirement; *cbtA*, *cbtB* - dicarboxylate binding protein; *dctA*, *dctB* - dicarboxylate transport (cytoplasmic membrane components); *omp* - outer membrane protein; *galE* - UDPgalactose 4-epimerase; *aroB*, *argG*, *metB*, *his*, *leu* - aromatic amino acid, arginine, methionine, histidine, and leucine requirements respectively.

As well, galE mutants are unable to ferment, and are sensitive to growth on, galactose (92). The galE revertant strain SK11R was selected by its ability to grow on glycerol in the presence of galactose.

5.2.2 Growth of Cells

Composition of growth media was outlined in Section 2.2.2. For binding and transport studies, the bacterial strains were grown on LB as described in Chapter 2. Transport studies were carried out as outlined in Chapter 2.

5.2.3 SEP Binding Studies

Unless indicated otherwise, SEP binding studies were carried out in 50 mM phosphate buffer (pH 7.5) at room temperature in silanized Eppendorf microfuge tubes. Binding assays were initiated by the addition of [^{35}S]-labelled SEP to the cell suspension. The final volume and cell density were 1.0 ml and 3.5×10^8 cells/ml, respectively. After 15 min, cells were pelleted in a microcentrifuge. The amount of radioactivity associated with the cell pellet was determined by first dissolving the pellet in 1 ml of 10% SDS at 75°C. The whole microfuge tube was then counted in the presence of 14 ml of scintillation fluid. All experiments were carried out in triplicate. The amount of nonspecific binding was determined by binding studies with porin$^-$ mutant strain JW031. It will be shown later that JW031 cannot bind specifically with SEP; thus this strain can be used to determine the amount of SEP nonspecifically bound to the cell surface and to the microfuge tubes, and trapped in the cell pellet.

5.2.4 Outer Membrane Protein Profiles

For visualization of outer membrane protein profiles, cells were grown overnight in 10 ml of LB, washed in 5 ml of 20 mM potassium

phosphate (pH 7.0), resuspended in 4 ml of phosphate and sonicated in ice (using a Branson sonifier, with a 70% pulse cycle) until clarified. Unbroken cells were removed by a 10 min spin at 3,000 x g. 4 ml of a solution containing 2% Triton X-100, 10 mM $MgCl_2$, and 40 mM Tris-HCl, pH 7.4 was added to the crude extract. The suspension was kept at 37°C for 1 hr to solubilize the cytoplasmic membrane components. The outer membrane was pelleted by centrifugation at 100,000 x g for 35 min. The pellet was washed once and resuspended in 1.0 ml of phosphate; an 80 µl aliquot was removed and the proteins precipitated with 10% (final) trichloroacetic acid (TCA) on ice for 1 hr. The pellet was washed twice with acetone to remove TCA, and the acetone was allowed to evaporate.

As SDS polyacrylamide gel electrophoresis did not resolve the different porins (especially when more than one species was present) adequately enough for quantitation, isoelectric focusing according to Sato and Yura (93) was performed. 120 µl of lysis buffer (8 M urea, 2% Triton X-100, 5% 2-mercaptoethanol, 1.6% ampholytes (pH 4 to 6)(LKB) and 0.4% ampholytes (pH 3.5-10)) was used to resuspend the acetone washed pellet. Solubilization consisted of three freeze-thaw cycles after which the sample was spun in a microfuge for 2 min to get rid of particulate matter. An 80 µl sample was loaded onto a tube gel (2.5 mm diameter) containing 4% acrylamide, 8 M urea, 2% Triton X-100, 1.6% ampholytes (pH 4-6), and 0.4% ampholytes (pH 3.5-10). The sample was overlaid with 10 µl of sample overlay buffer containing 4 M urea and 0.5% ampholytes. The upper and lower reservoirs were filled with 0.02 M NaOH and 0.03 M H_3PO_4, respectively. Electrophoresis was carried out at 400 V for 12 hr and 600 V for a further 2 hours. The gels were fixed and washed (2.5 hr each) in 30% ethanol, 7% acetic acid to remove

ampholytes, stained in Coomassie blue, and destained in 10% methanol, 5% acetic acid. Scanning of gels was carried out in a Beckman DU-8 UV/VIS spectrophotometer using "Gel Scan" software with automatic area calculation.

5.2.5 Preparation of Antibodies to OmpC and OmpF

To facilitate identification of strains by Western (immuno) blotting, antibodies to porin OmpF and to porin OmpC were prepared. Porin was isolated from CBT43, grown in LB so that both OmpF and OmpC were produced. The NaCl-SDS porin isolation procedure is outlined in Section 6.2.1.1 and 6.2.1.2. The protein was run on 12% denaturing SDS-PAGE gels according to Laemmli (59) to separate OmpC from OmpF. The protein was applied as a continuous band across the top of the gel. The gel was run for 2 hr after the dye front had migrated off the end (to maximize the separation between OmpC and OmpF) and was then stained in Coomassie blue. After destaining, the gel was dried (to more clearly define the bands), and the OmpF and OmpC protein bands cut out. The backing paper was removed, and the gel was macerated with a razor blade, and homogenized in sterile water in a Teflon homogenizer (Thomas). From 3 gels the final volume of gel homogenate was 8 ml, 2 ml of which was used for injecting (without any additional adjuvent) into the thigh of a female albino rabbit. Adjuvent is unnecessary because of the slow release of protein from the acrylamide. The remaining solution was stored frozen at -20°C. The rabbits were injected biweekly for 6-8 weeks and bled 7 days after the last injection. The serum was complement inactivated at 55°C for 30 min, and stored at -20°C.

5.2.6 Western (Immuno-) Blotting of Strains

Anti-OmpC and anti-OmpF antisera were used in an attempt to distinguish between strains used for the whole cell binding studies. Triton extracted, sonicated cell envelopes were run on 11% SDS-PAGE gels. Proteins were electrophoretically transferred onto nitrocellulose (81) as outlined in Section 4.2.5. The nitrocellulose was equilibrated with Tris-buffered saline (TBS, pH 7.4). The nitrocellulose was cut into strips, and nonspecific protein binding sites were blocked with 4% bovine serum albumin in TBS (BSA-TBS, pH 7.4) for 2 hr at room temperature. Anti-OmpC or OmpF antiserum was added and incubated at room temperature for 4 hours. The nitrocellulose was washed six times with TBS and 50,000 cpm of [^{125}I]-protein A in BSA-TBS was added to each lane. After overnight incubation, the nitrocellulose was washed six times in TBS, dried, and exposed to Kodak X-OMat AR film, in the presence of a Dupont Cronex intensifying screen at -70°C.

5.3 RESULTS

5.3.1 Binding of SEP to Intact Whole Cells

To investigate the interaction of SEP with cell surface components, radioactively labelled SEP was prepared from cells grown in the presence of [^{35}S]-sulfate. The isolation and purity of the SEP was as shown in Chapter 2 (i.e. protein was eluted with succinate and desalted on Bio-Gel P-6DG). LB grown exponential cells were used in the binding assays. Fig. 5.1 shows the binding of SEP to two different bacterial strains. Previous work by Bewick and Lo (45) had shown BL25 was defective in DBP; thus more sites may be available for SEP binding. Strain JW031 was an *ompF* and *ompC* mutant. As will be shown below, this strain cannot bind

FIGURE 5.1

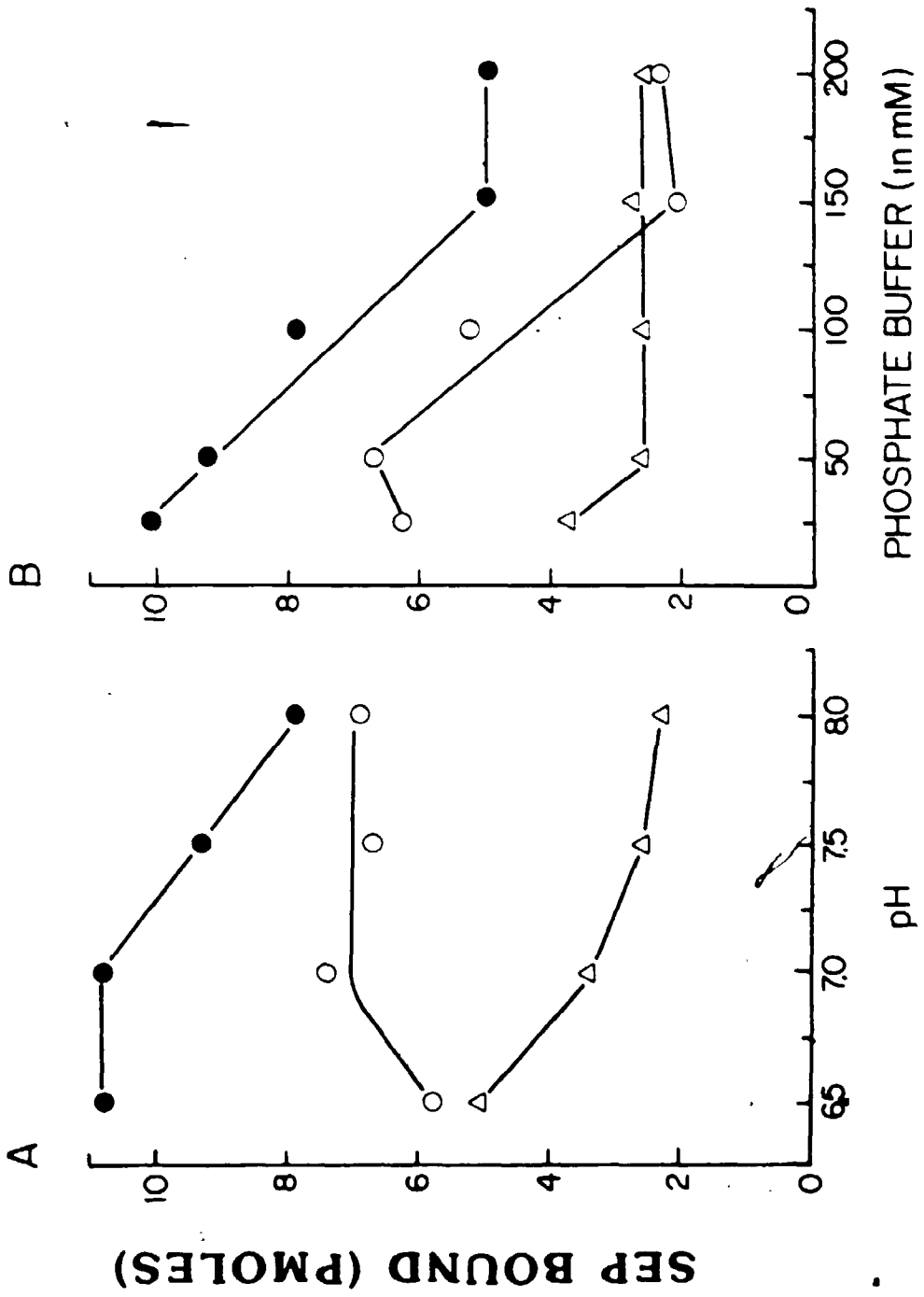
SEP BINDING TO INTACT WHOLE CELLS

Binding assays were carried out as described in "Experimental Procedures". In each assay, 39 pmoles of SEP (osmotic shock released) and 3.5×10^8 cells were used. The concentration of SEP was 43 ug/ml, and the specific activity was 1000 dpm/7.7 pmole. Figure 5.1A indicates SEP binding at different pH values. 50 mM sodium phosphate buffers were used in these studies. Figure 5.1B shows the effect of ionic strength on SEP binding. These studies were carried out in sodium phosphate buffer, pH 7.5.

(●) - Binding of SEP to strain BL25.

(△) - Binding of SEP to strain JW031

(○) - Net binding (the difference betwen BL25 and JW031)


SEP BOUND (PMOLES)
A
B
pH
PHOSPHATE BUFFER (in mM)
0
2
4
6
8
10
6.5
7.0
7.5
8.0
50
100
150
200

specifically with SEP; and radioactivity associated with JW031 is an indication of nonspecific binding and trapping. The difference in radioactivity associated with BL25 and JW031 should reflect the amount of specific binding. As shown in Fig. 5.1A, maximum specific binding of SEP occurred from pH 7.0 to 8.0. There was a considerable amount of nonspecific binding at lower pH values. Increasing the ionic strength of the phosphate buffer (pH 7.5) also affected the amount of specific binding (Fig. 5.1B). Optimal binding occurred at a phosphate concentration of 50 mM.

5.3.2 Effect of Various Ions on SEP Binding

Various monovalent and divalent ions and chelating agents were tested for their effect on SEP binding to BL25 and JW031. It can be seen (Table 5.2) that low concentrations of the chelating agents EGTA and EDTA had little effect on the net (specific) binding. The presence of Cl^- ions (in KCl, NaCl and $MgCl_2$) increased nonspecific binding. A very marked increase in nonspecific SEP binding is seen in the presence of $CuSO_4$, however the change in net binding was minimal. $MgCl_2$ and $MgSO_4$ caused a 40–50% reduction in net binding. The most dramatic reductions in net binding were caused by the divalent cations, Tris, Ca^{++}, and Zn^{++}. There was both a slight increase in binding to JW031 (possibly due to the presence of Cl^-) as well as a 50% reduction in the SEP bound to BL25.

5.3.3 Effect of Dicarboxylic Acids on SEP Binding

Dicarboxylic acids at 5 and 10 μM concentration were tested for their effect on SEP binding. As can be seen in Table 5.3 there was no significant effect of the substrates.

TABLE 5.2

EFFECT OF VARIOUS IONS ON BINDING OF SEP TO WHOLE CELLS

ION		BINDING TO BL25 (%)	JW031 (%)	NET BINDING (%)
Control		100 ± 2	100 ± 4	100 ± 2
NaCl	1 mM	153 ± 18	227 ± 4	115 ± 28
KCl	1 mM	157 ± 29	230 ± 36	119 ± 48
EDTA	0.1 mM	136 ± 6	241 ± 45	83 ± 25
EGTA	0.1 mM	125 ± 20	196 ± 19	88 ± 32
$ZnCl_2$	1 mM	50 ± 2	124 ± 16	13 ± 8
$CaCl_2$	1 mM	54 ± 3	132 ± 11	13 ± 8
$MgCl_2$	1 mM	112 ± 2	234 ± 3	49 ± 4
$MgSO_4$	1 mM	75 ± 18	102 ± 8	60 ± 10
$CuSO_4$	1 mM	208 ± 27	487 ± 25	139 ± 19
$(NH_4)_2SO_4$	1 mM	129 ± 3	121 ± 13	134 ± 8
Tris-HCl	50 mM	52 ± 5	150 ± 13	1.6 ± 11

Binding of SEP to whole cells in the presence of various ions was carried out as outlined in "Experimental Procedures". Binding of SEP is expressed as a percentage of the "Control". Binding to the control was carried out in the presence of 50 mM phosphate, pH 7.5. "Net Binding" is the difference between binding to the porin containing BL25 and the porin-less strain JW031. Under the "Control" conditions 7.7 pmoles of SEP/3.5 x 10^8 cells was bound to BL25, and 2.6 pmoles was bound to JW031.

TABLE 5.3

EFFECT OF DICARBOXYLIC ACIDS ON BINDING OF SEP TO WHOLE CELLS

SUBSTRATE		BINDING TO BL25 (%)	JW031 (%)	NET BINDING (%)
EXPERIMENT 1:				
Control		100 ± 8.5	100 ± 5	100 ± 24
Succinate	5 μM	114 ± 3	84 ± 5	138 ± 34
	10 μM	102 ± 13	110 ± 11	113 ± 22
Malate	5 μM	96 ± 10	114 ± 6	101 ± 38
	10 μM	124 ± 4	134 ± 5	118 ± 32
Fumarate	5 μM	116 ± 2	118 ± 1	121 ± 20
	10 μM	126 ± 1	112 ± 13	147 ± 11
d-Lactate	5 μM	120 ± 4	106 ± 3	145 ± 17
	10 μM	132 ± 16	94 ± 7	172 ± 27
Aspartate	5 μM	108 ± 8	104 ± 2	126 ± 13
	10 μM	99 ± 4	96 ± 47	116 ± 33
Glutamate	5 μM	95 ± 4	77 ± 5	119 ± 7
	10 μM	92 ± 2	64 ± 25	121 ± 13
EXPERIMENT 2:				
Control		100 ± 7	100 ± 8	100 ± 16
Succinate	5 μM	100 ± 11	71 ± 11	115 ± 18
	10 μM	107 ± 11	79 ± 40	120 ± 30
d-Lactate	5 μM	77 ± 7	53 ± 19	90 ± 16
	10 μM	80 ± 5	93 ± 12	78 ± 20
Glutamate	5 μM	99 ± 10	113 ± 10	87 ± 17
	10 μM	72 ± 21	97 ± 14	61 ± 36

Binding of SEP to whole cells was done in the presence of various substrates and analogues. The amount of binding is expressed as a percentage of the SEP bound to cells in the absence of substrate. "NET BINDING" is the difference in the amount bound to BL25 and the porin-less JW031 strain. In Experiment 1 the amount of SEP bound to control BL25 was 7.8 pmoles/3.5 x 10^8 cells, and to JW031 was 2.5 pmoles/3.5 x 10^8 cells. In Experiment 2 6.2 and 2.5 pmoles were bound respectively.

5.3.4 Porin is Involved in SEP Binding

In order to determine the outer membrane protein(s) responsible for SEP binding, various outer membrane mutants were analysed. The profiles of the OmpC, OmpF, and PhoE proteins in various sets of isogenic strains are shown in Fig. 5.2. Only a residual level of the OmpF protein was observed in various parental strains under our growth conditions (LB medium containing 170 mM NaCl). By scanning the gels with a densitometer, the OmpC/OmpF ratios were found to be 23:1, 4:1, and 11:1 for the parental strains VR42, CBT43, and JF568 respectively. Similar findings were observed by van Alphen et al (94). Fig. 5.2 also shows that the OmpC protein is absent in strains JF701, and the OmpF protein is missing in strain JF703. JW031 is missing both OmpC and OmpF.

Fig. 5.3 shows SEP binding to three isogenic strains and BL25. The parental strain (VR42) and BL25 contain OmpA and porins whereas these proteins are absent in strains PL6 and JW031, respectively. This figure shows that strains VR42 , PL6, and BL25 can bind with substantial amounts of SEP, whereas strain JW031 cannot. This indicates that porin is necessary for binding of SEP to the cell surface, whereas OmpA is not required.

5.3.5 Porin Species and Succinate Transport

Lo and Bewick (47) demonstrated that, at 2 μM succinate, the transport activity of JW031 (porin$^-$), but not that of PL6 (OmpA$^-$), was impaired compared to the parental VR42 (porin$^+$,OmpA$^+$). At higher concentrations all three isogenic strains took up succinate at similar rates, suggesting porin is only required at low succinate concentrations. Our preliminary experiments using these mutants were in complete agreement with these original results.

FIGURE 5.2

ISOELECTRIC FOCUSING POLYACRYLAMIDE GELS SHOWING THE PRESENCE OF PORINS IN VARIOUS BACTERIAL STRAINS

Outer membrane proteins were prepared and gel electrophoresis was carried out according to the method of Sato and Yura (93) as described in "Experimental Procedures". Protein bands were stained by Coomassie blue. Only the lower pH end of the gel is shown.

Lane (1) CBT43; (2) BL3; (3) BL25; (4) VR42; (5) JW031; (6) PL6;

(7) JF568; (8) JF701; (9) JF703; (10) 4498; (11) SK11; (12) SK11R.

FIGURE 5.3

SEP BINDING TO VARIOUS OUTER MEMBRANE MUTANTS

Binding assays were carried out as described in the legend to Fig. 5.1. The amount of nonspecific binding was not subtracted from the data.

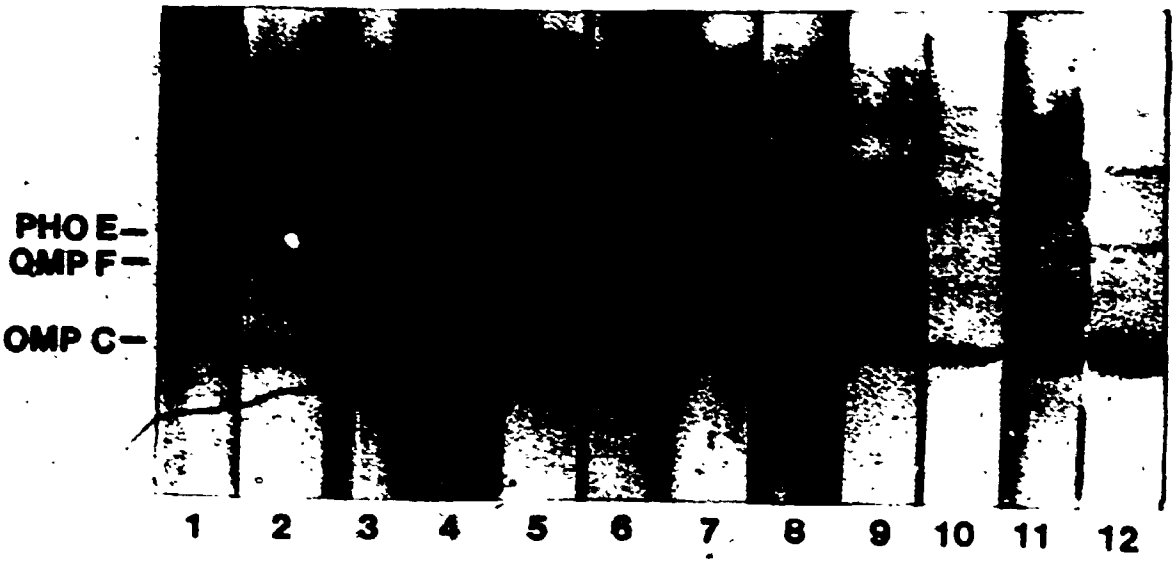
The bacterial strains were:

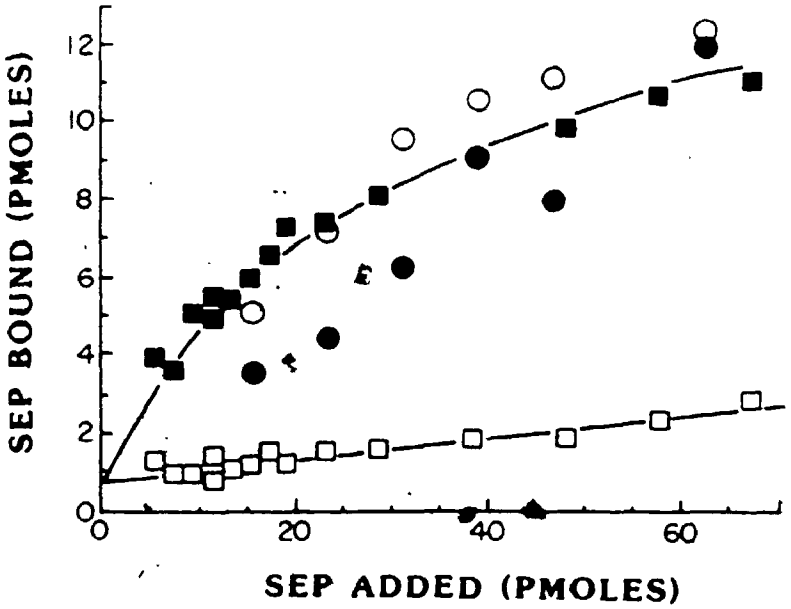
(○) - VR42 ($OmpC^+$, $OmpF^+$, $OmpA^+$)

(●) - PL6 ($OmpC^+$, $OmpF^+$, $OmpA^-$)

(□) - JW031 ($OmpC^-$, $OmpF^-$, $OmpA^+$)

(■) - BL25 ($OmpC^+$, $OmpF^+$, $OmpA^+$)


PHO E—
OMP F—
OMP C—
1
2
3
4
5
6
7
8
9
10
11
12


SEP BOUND (PMOLES)
0
2
4
6
8
10
12
0
20
40
60
SEP ADDED (PMOLES)

To determine whether any particular species of porin could preferentially transport succinate we examined succinate uptake in JF701, JF703, and JF694 at 2 μM succinate. Strain JF568 is the parental strain while the mutants each produce a single species of porin. JF701 produces OmpF, JF703 produces OmpC and JF694 produces 'phosphoporin' (PhoE). It can be seen (Fig. 5.4) that uptake by the PhoE producing strain is very much higher than uptake by OmpC and OmpF producing strains. This is in agreement with other work on PhoE. This protein, which is induced for the uptake of negatively charged phosphate, is thought to contain a positively charged amino acid side chain within its pore to aid in the transport of negatively charged compounds (95). OmpC (JF703) seems to transport succinate somewhat better than OmpF (JF701). This is in agreement with evidence indicating that OmpF shows some degree of preference for cationic compounds (96). The reason for the slower uptake by JF568, which contains largely OmpC but has uptake similar to JF701, is not clear, although the difference is not that significant.

Fig. 5.5 shows SEP binding to the JF-series of isogenic porin mutants. It can be seen that all strains bind SEP although OmpC and OmpF seem to bind a greater amount than PhoE does. The above data indicates that strains producing porins can interact with SEP.

5.3.6 Comparison of Antibody Binding

Antibodies prepared against denatured OmpF and OmpC isolated from CBT43 were used to determine the structural homology between OmpC, OmpF and PhoE. Outer membranes from the various strains used for binding were run on SDS-PAGE gels and probed by Western blotting.

FIGURE 5.4

SUCCINATE UPTAKE BY PORIN MUTANTS

Transport studies were performed as described in the Section 2.2.7 using 2 uM succinate. Transport studies were carried out in the same buffer (50 mM sodium phosphate, pH 7.5) as in the binding assays. The specific activity of the [^{14}C]-succinate was 1000 dpm/9.45 pmoles.

The bacterial strains used were:

(●) - JF568 ($OmpC^+$, $OmpF^+$)

(○) - JF694 ($OmpC^-$, $OmpF^-$, $PhoE^+$)

(□) - JF701 ($OmpC^-$, $OmpF^+$)

(△) - JF703 ($OmpC^+$, $OmpF^-$)

FIGURE 5.5

SEP BINDING TO PORIN MUTANTS

SEP binding assays were carried out as described in Figure 5.1, except that the amount of nonspecific binding was subtracted from the data. The amount bound to JW031 was used as the background counts.

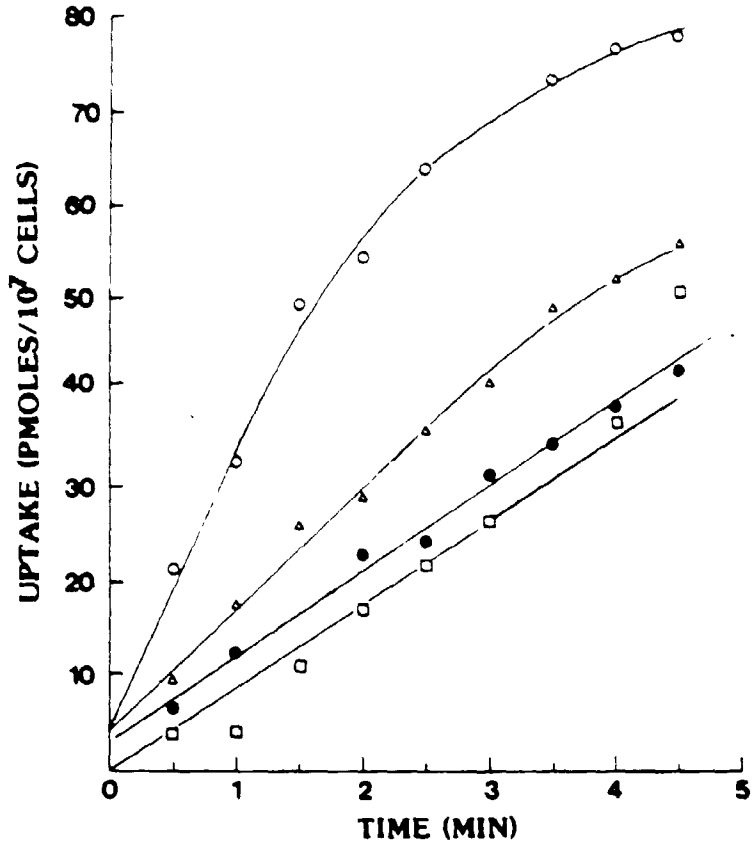
The bacterial strains used were:

(▲) - JF568 ($OmpC^+$, $OmpF^+$)

(▽) - JF694 ($OmpC^-$, $OmpF^-$, $PhoE^+$)

(□) - JF701 ($OmpC^-$, $OmpF^+$)

(○) - JF703 ($OmpC^+$, $OmpF^-$)


UPTAKE (PMOLES/10⁷ CELLS)
80
70
60
50
40
30
20
10
0
1
2
3
4
5
TIME (MIN)

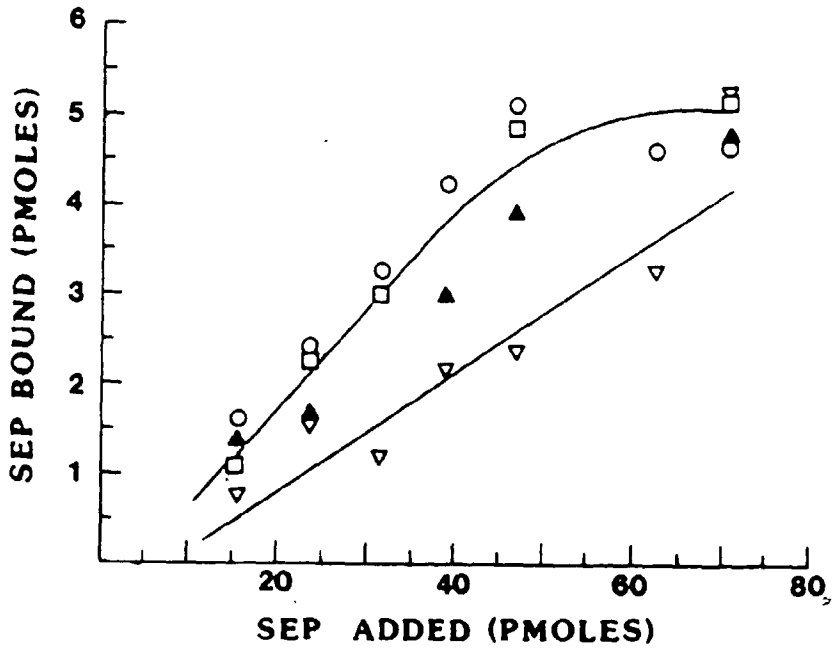

SEP BOUND (PMOLES)
6
5
4
3
2
1
20
40
60
80
SEP ADDED (PMOLES)

FIGURE 5.6

WESTERN BLOTTING USING ANTI-PORIN ANTIBODIES

Outer membrane proteins from various strains were separated by SDS-PAGE, blotted onto nitrocellulose; and probed with antibodies prepared against SDS-PAGE separated OmpF and OmpC as described in Section 5.2.6. An equal amount of protein was added in each lane. Visualization of bound antibodies was by ^{125}I-protein A and autoradiography.

Panel A shows reaction of anti-OmpF with one preparation of membranes. Panel B and C show anti-OmpF and anti-OmpC reactions with a second preparation.

Lane 1 = JF568 - parental; produces mainly OmpC

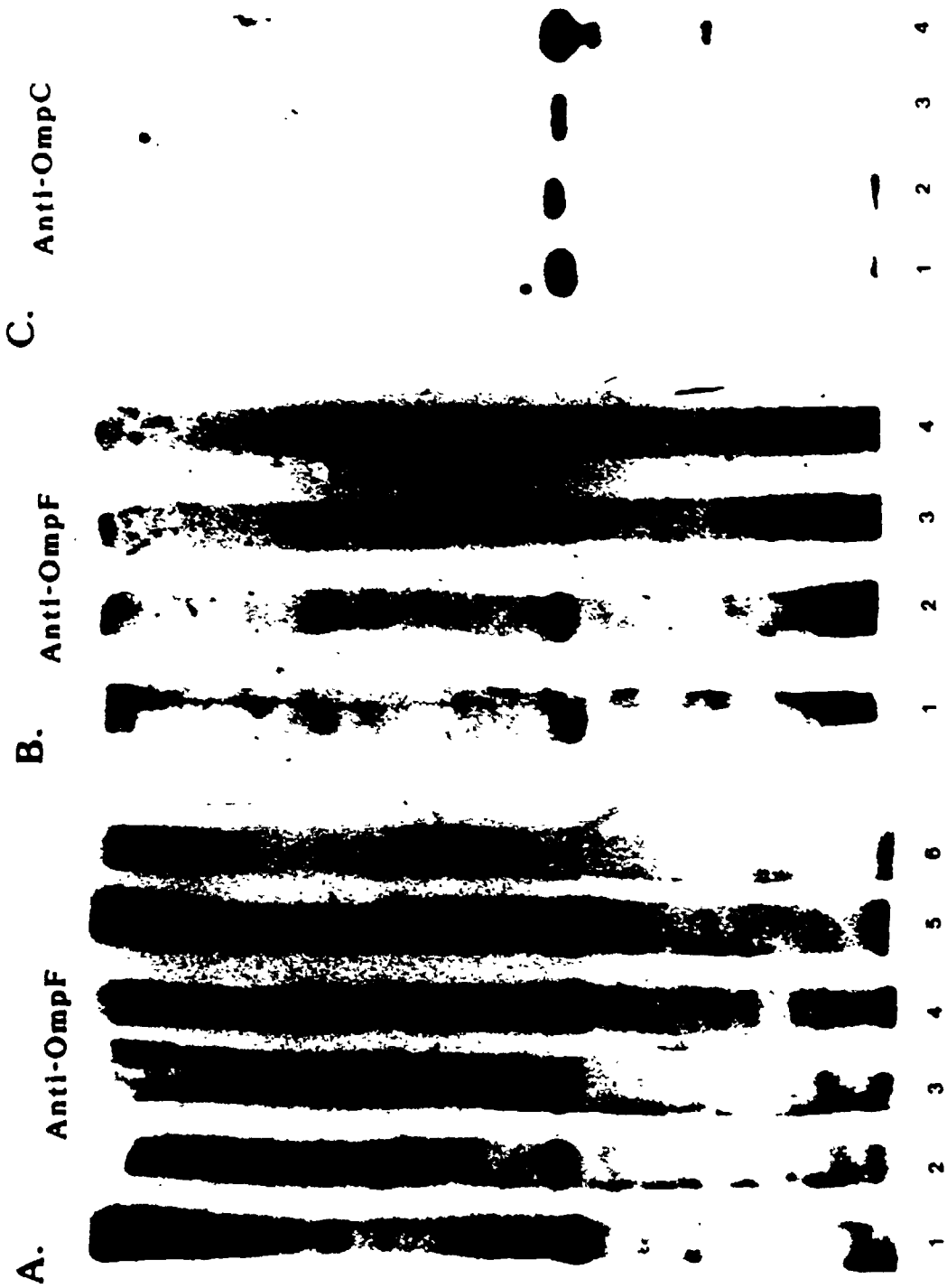
2 = JF694 - phosphoporin ($PhoE^+$)

3 = JF701 - $OmpF^+$

4 = JF703 - $OmpC^+$

5 = PL6 - Wild type porins, $OmpA^-$

6 = JW031 - $porin^-$, $OmpA^+$


A.
Anti-OmpF
1
2
3
4
5
6
B.
Anti-OmpF
1
2
3
4
C.
Anti-OmpC
1
2
3
4

As can be seen (Fig. 5.6) anti-OmpC and anti-OmpF recognize each of the species of proteins, indicating considerable homology. The anti-OmpF reacts with all the species equally while the anti-OmpC shows a bit more specificity in that OmpF or PhoE are not as strongly recognized. Because of the high degree of homology between the porins in terms of immunologically related protein domains, the fact that SEP can interact with all species of porin is not unexpected.

5.3.7 SEP Binding Capacity and Affinity of Intact Whole Cells

Since some of the porin is normally occupied by endogenous cell surface DBP, the number of sites available for binding to exogenously added SEP may vary with different cbt mutants. Fig. 5.7A shows SEP binding to three isogenic strains. The parental strain (CBT43) is assumed to contain the "normal" level of cell surface DBP. Scatchard analysis (Fig. 5.7B) of the data indicates that CBT43 has a binding capacity of 10.5 picomoles per 3.5×10^8 cells, or 18×10^3 molecules per cell. Work by Bewick and Lo has indicated that the cbtB mutant (BL25) is defective in all three forms of DBP, whereas the DBP in the cbtA mutant (BL3) is defective in the "transport component recognition site" (TRS) (45). Fig. 5.7A shows that both strains have higher SEP binding capacity. Scatchard analysis of SEP binding to BL25 indicates that each bacterium can bind with 30×10^3 molecules of exogenously added SEP. The Scatchard plots also show that the SEP binding affinity is around 30 nM for both CBT43 and BL25.

5.3.8 Effect of Cell Surface Alterations

Fig. 5.8 shows the binding of SEP to two non-isogenic galE mutants. The lipopolysaccharide in these mutants has very short polysaccharide side chains and consequently the mutants would be expected to have

FIGURE 5.7

SEP BINDING TO DBP MUTANTS

(A) Binding to Whole Cells.

SEP binding experiments were carried out as described in Figure 5.5, with subtraction for nonspecific binding.

The bacterial strains used were:

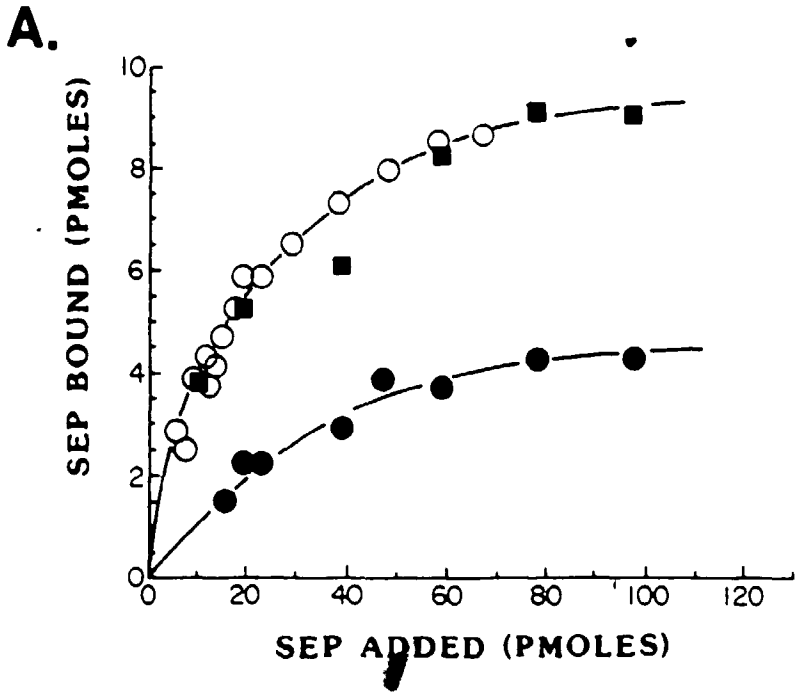
(●) - CBT43 (parental)

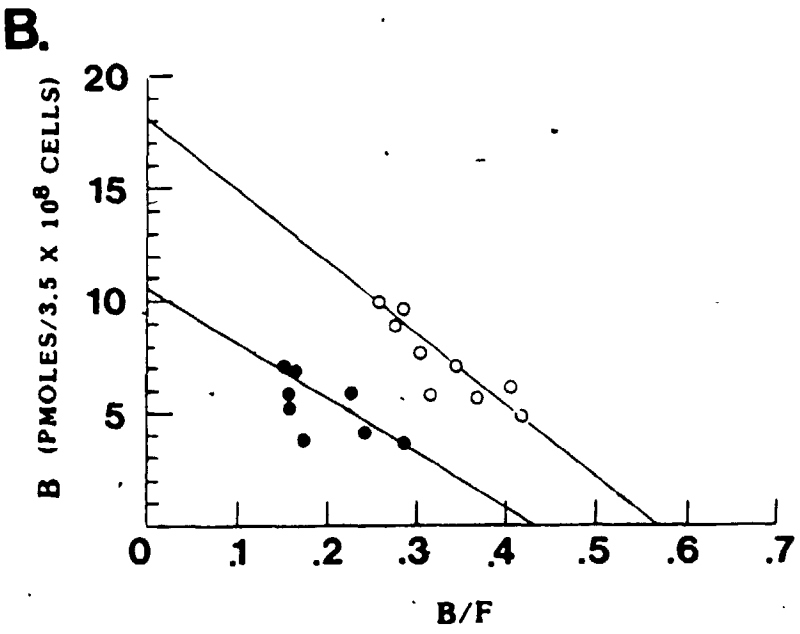
(■) - BL3 (*cbtA*)

(○) - BL25 (*cbtB*)

(B) Scatchard Plot of SEP Binding.

SEP binding to CBT43 (●) and BL25 (○) was plotted according to the method of Scatchard (97) and the lines were fitted by hand. B represents the amount of SEP bound, F represents the amount of free SEP remaining in solution. B_{max} can be obtained from the Y-intercept. For CBT43 it is 10.5 pmoles/3.5 x 10^8 cells and for BL25 it is 18 pmoles/3.5 x 10^8 cells. K_D can be derived from the X-intercept. For CBT43 it is 28 nM, and for BL25 it is 32 nM.


A.
SEP BOUND (PMOLES)
0
2
4
6
8
10
0
20
40
60
80
100
120
SEP ADDED (PMOLES)


B.
B (PMOLES/3.5 X 10^8 CELLS)
20
15
10
5
0
.1
.2
.3
.4
.5
.6
.7
B/F

altered cell surface properties (91). This figure shows that SEP is unable to bind to the cell surface of the galE mutants (SK11 and 4498). Fig. 5.2 shows that these mutants contain the normal amount of OmpC protein. Thus the lack of interaction between SEP and the cell surface may be attributed to the alteration of the lipopolysaccharide.

Fig. 5.8B shows that both galE mutants are unable to take up 2 uM succinate; whereas the galE revertant (SK11R) regains transport activity. Thus the transport defect may be due to the lack of interaction between SEP and porin. It is possible that alteration in lipopolysaccharide may result in conformational changes of the porin, and this consequently affects the interaction between SEP and porin.

5.3.9 SEP Binding to Dicarboxylic Acid Transport Mutants

Some of the dicarboxylic acid transport mutants available in the laboratory were thought to be defective at the level of the cytoplasmic membrane transport components (78). When SEP binding to these mutants was examined, one of them, LL5, was unable to bind SEP (Fig. 5.9). This indicates that the transport defect in LL5 may be a cell surface defect rather than a cytoplasmic membrane defect. As porin levels were normal in this strain, the possibility exists that this may be an LPS mutant, however more experiments have to be carried out to verify this.

5.4 DISCUSSION

Bewick and Lo (45-47) have previously shown that a population of protein (identified as DBP by its ability to bind to aspartate-Sepharose) is present on the cell surface. This cell surface protein was thought to play an important role in the transport of dicarboxylic acids across the outer membrane at low substrate concentrations. At

FIGURE 5.8

SEP BINDING AND TRANSPORT PROPERTIES OF *galE* MUTANTS

SEP binding and transport studies were performed as described in the text. Transport studies were carried out with 2 uM succinate. Panels A and B show binding and transport data respectively.

The bacterial strains used were:

(■) - CBT43 ("wild type")

(○) - SK11 (*galE*)

(●) - SK11R (*galE*$^+$ revertant of SK11)

(□) - 4498 (*galE*)

FIGURE 5.9

SEP BINDING TO DICARBOXYLATE TRANSPORT MUTANTS

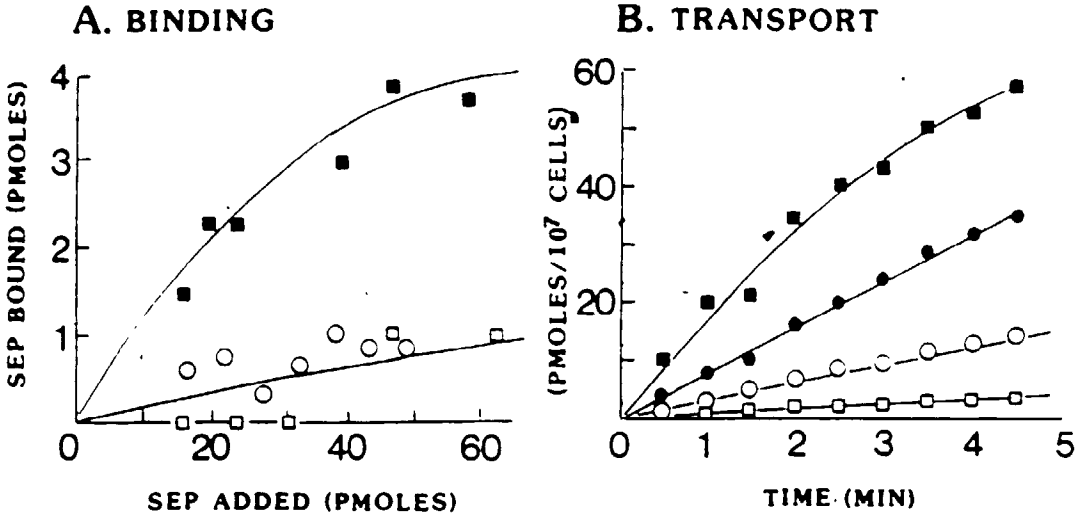
SEP binding assays were carried out as described in Figure 5.5, with subtraction of nonspecific binding.

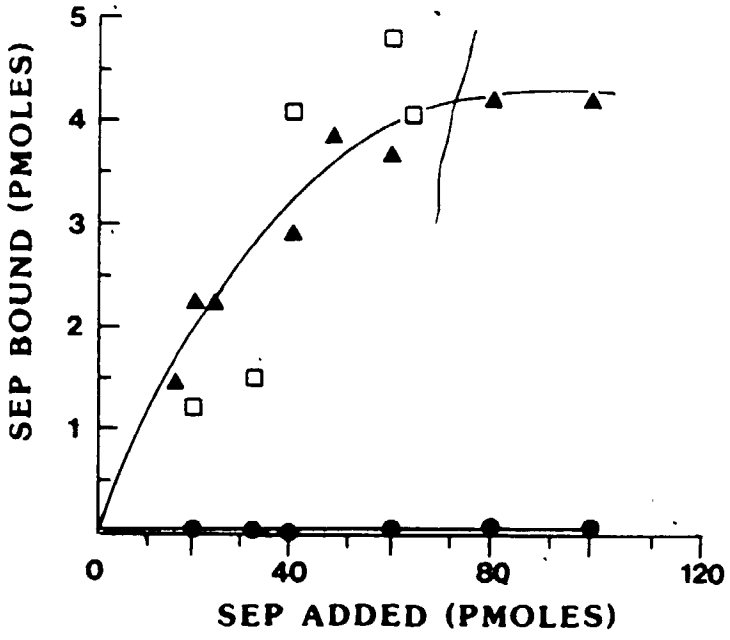
The bacterial strains used were:

(▲) - CBT43 (parental)

(□) - LL3 (*dctA*)

(●) - LL5 (*dctB*)


A. BINDING
SEP BOUND (PMOLES)
4
3
2
1
0
20
40
60
SEP ADDED (PMOLES)
B. TRANSPORT
(PMOLES/10⁷ CELLS)
60
40
20
0
1
2
3
4
5
TIME (MIN)


SEP BOUND (PMOLES)
5
4
3
2
1
0
40
80
120
SEP ADDED (PMOLES)

these low substrate concentrations, dicarboxylate transport is also dependent on the presence of porin (47).

SEP is a major cell envelope protein that was shown to stimulate succinate transport when added externally. Several lines of evidence presented in this chapter point to an interaction between SEP and the cell surface. The results indicate that externally added, radioactively labelled, SEP can bind to intact whole cells. Scatchard plot analysis of the interaction indicates that the binding was taking place with a high affinity (K_D of about 30 nM) and about 30,000 molecules/cell were maximally bound. The presence of normal porin and LPS in the outer membrane was required for such binding. Mutants lacking porin, but containing the other major outer membrane protein, OmpA, failed to bind SEP. Mutant cells with defective LPS (galE mutants) also failed to bind SEP. The presence of low concentrations of dicarboxylic acids did not affect the interaction while divalent cations had a deleterious effect.

The species of porin present did not seem to be of particular importance to SEP binding , as mutants expressing OmpC, OmpF, or PhoE all bound SEP. These various porin species exhibited a high degree of homology as shown by immunological cross-reactivity on Western blots. The species of porin present did have an effect on the rate of succinate uptake. Succinate uptake was observed as long as one of these porin species is present, but PhoE transported succinate at the highest rate, followed by OmpC and OmpF.

Cells with altered LPS were also defective in succinate transport even though OmpC was present. In the studies presented here, galE mutants, which have a shortened polysaccharide chains, did not bind externally added SEP or transport succinate while a revertant regained

the ability to transport succinate.

It is possible that LPS may be indirectly affecting SEP binding by altering the porin structure. LPS and porin exist in close contact (2) and isolation of porin generally entails the co-isolation of a tightly bound population of LPS (98, and Fig. 6.2). It is possible that the binding of SEP is dependent on the correct conformation of porin which is in turn dependent on the presence of normal LPS. The change in porin conformation could be radical, resulting in large scale rearrangements and affecting passage of all solutes. On the other hand the change may be more subtle, with slight changes in conformation at the cell surface leading to loss of the ability to bind SEP. Alternately the interaction between LPS and SEP may be direct rather than exerted through an effect on porin conformation, and a specific LPS structure may be recognized by SEP.

An analogy can be drawn between SEP binding to the cell surface and the interaction of bacteriophages with the cell surface. Bacteriophages often recognize specific proteins on the cell surface. Phages Tula, Tulb, and TC45 recognize OmpF, OmpC, and PhoE respectively. But LPS is also important in the phage interaction and may serve to maintain the correct confogmation or localization of the receptor (99). LPS by itself can also serve as a phage receptor.

Various metal ions are important components of the outer membrane (100). Monovalent cations, K^+ and Na^+ did not seem to have on effect on SEP binding. These ions have been shown to bind to isolated outer membranes but may be associated with phospholipids (101). Divalent cations are known to be important in maintaining the integrity of the outer membrane. They are thought to serve as ionic 'bridges' between

negatively charged porin (pI around 5) and negatively-charged LPS (2). Studies have indicated that in E. coli, calcium is important for the stabilization of LPS, while magnesium helps in the formation of intramembranous LPS-protein complexes (101). The reduction of SEP binding induced by divalent cations may be due to an effect on porin or LPS. Tris-HCl has been shown to affect the outer membrane of Gram-negative bacteria (74) and its action is thought to be due to the displacement of membrane located divalent cations. If a specific divalent cation is responsible for maintaining the conformation of porin in the outer membrane or is involved directly in SEP binding, the displacement of the specific ion by Tris, Ca^{++}, or Zn^{++} may lead to the observed reduction in SEP binding. Alternately, increased concentrations of divalent cations above those normally found may lead to general conformational changes (in porin or LPS) that are unfavourable for SEP binding.

The results of the investigation of SEP binding to various dicarboxylic acid transport mutants was interesting. Dct mutants (i.e. LL3) bound SEP at levels similar to the parental CBT43. This is expected as these mutations were thought to be in the cytoplasmic membrane transport components. There does not seem to be an involvement with the outer membrane. One of the transport mutants, LL5, demonstrated lack of SEP binding (i.e. a level comparable to JW031 or SK11). This strain shows normal levels of porin (78) so the mutation may be in LPS although further work has to be done to verify this. It is possible that SEP binding could be used as a diagnostic tool to determine if an outer membrane defect (which does not show up in protein

profiles) or cytoplasmic membrane defect is involved in the dicarboxylic acid transport mutants.

On the other hand, mutants that are thought to be defective in DBP (BL3, BL25) are found to bind with more SEP than the parental strain. The absence of cell surface DBP may mean that more sites are available to bind to the externally added SEP.

The increased succinate transport activity induced by the addition of SEP to whole cells, together with the observation that transport activity cannot be detected in cells that are unable to bind with SEP is evidence for the involvement of cell surface SEP in dicarboxylic acid transport in vivo. A possible mechanism may involve blocking charged groups on the cell surface around the porin channel. Charged groups on porin are important for transport. This is demonstrated by the preferential transport of succinate by PhoE, a protein evolved for the transport of negatively charged phosphate compounds (95). PhoE contains positively charged amino acid residues within its channel. Various other workers have modified the transport properties of OmpC and OmpF by chemically altering exposed amino acid residues on the surface and within the channel (1,96). These modifications have resulted in altered transport rates through the pores. (For example, amidination of negatively charged carboxyl groups of OmpF resulted in loss of cation selectivity). It is possible that SEP could increase transport of the negatively charged succinate by blocking negatively charged residues on the cell surface. Perhaps the binding of SEP with aspartate-Sepharose is simply a reflection of its ability to bind to negatively charged surfaces. Under the conditions that were used to measure succinate uptake and SEP binding (i.e. pH 7.5) one would expect the succinate and

surface macromolecules to be deprotonated, and the charge considerations to be especially important for transport.

If SEP is not directly involved in succinate transport, the reason for the increased binding of externally added SEP to BL25 in comparison to CBT43 is not clear. As shown in the preceeding chapter both strains produce SEP although the levels of the other major periplasmic protein (the 52K protein) are considerably reduced in BL25. The 52K protein may bind to whole cells in a manner analogous to SEP. (It is certainly present in the EDTA-sucrose releasable fraction of CBT43.) Absence of this protein at the cell surface would mean that more sites are available for binding of exogenously added SEP, if the proteins share the same binding sites. On the other hand, BL25 was shown to be defective in DBP production (45). If SEP and DBP share the same binding sites on the cell surface, the absence of DBP would mean more sites are available for SEP binding.

In conclusion, we have shown that SEP binds to the cell surface of E. coli K12 and the ability of cells to bind SEP correlates well with their ability to transport succinate, provided the other components of the dicarboxylate transport system are present.

CHAPTER 6

SEP BINDING TO IMMOBILIZED SEPHAROSE

6.1 INTRODUCTION

Results presented in Chapter 5 indicated an involvement of porin and LPS in the interaction of SEP with whole cells. Porin and LPS mutants failed to bind SEP.

The interaction between an outer membrane protein and a binding protein was studied *in vitro* in the case of maltose transport. As outlined in Chapter 1, a number of experiments have implicated the maltose binding protein (MBP) in facilitating transport across the outer membrane. The interaction between MBP and maltoporin, if it does occur *in vivo*, presumably takes place on the inner side of the outer membrane. Ultrastructural localization studies indicated that MBP was located in close proximity to the outer membrane (25). However, externally added anti-MBP did not affect maltose transport (102). *In vitro* studies by Baovil and Nikaido (26) showed that maltoporin from detergent solubilized outer membranes, bound with MBP coupled to Sepharose. Elution of the bound maltoporin required high salt concentrations.

To better understand this SEP-porin interaction, we decided to look at the binding of radioactive SEP to isolated porin coupled to Sepharose. Because it was much easier to isolate milligram quantities of porin than SEP, we chose to couple the membrane component to Sepharose. As the methodology for coupling protein components to inert supports is better defined than methodology for coupling LPS, we decided to look at immobilized porin rather than immobilized LPS.

6.2 EXPERIMENTAL PROCEDURES

Growth of cells, and preparation of SEP were as described in Chapter 2. CBT43 was used for the preparation of SEP and porin (unless otherwise noted). The description of the JF-series of porin mutants is provided in Table 5.1. Protein concentration was determined by the method of Lowry et al (80). Cyanogen bromide was from Sigma, and Sepharose 4B was from Pharmacia.

6.2.1 Preparation of Porin-Sepharose

6.2.1.1 Preparation of Total Membrane Fraction

CBT43 grown in LB was used for the preparation of porin. A crude total membrane fraction was prepared by passing the washed cells in 50 mM sodium phosphate buffer (pH 8.0) three times through a French Pressure Cell (Aminco) at 10,000 p.s.i.

The turbid suspension was spun at 5,000 x g for 10 min to get rid of any unbroken cells. The supernatant was then spun at 100,000 x g for 30 min to pellet the cell membranes. The membranes were washed once in Tris-HCl buffer (20 mM, pH 8) and stored at -20°C until used.

6.2.1.2 Isolation of Porin from Total Membranes

Isolation of porins from the total membrane fraction was similar to the procedure of Tokunaga et al (103). 10% SDS was added to the thawed membrane suspension to a final concentration of 2%. The turbid yellowish solution immediately clarified. The cytoplasmic membrane and non-peptidoglycan associated outer membrane proteins were solubilized by incubation at 55°C for 30-45 min. After centrifugation at 100,000 x g for 30 min, the white pellet was washed once in 2% SDS, 20 mM Tris (pH 8.0). The porin was solubilized by resuspending the pellet in a solution containing 2% SDS, 0.5 M NaCl, 10 mM Tris (pH 8.0), and incubating

for 30 min at 37°C. The porin which is tightly associated with the peptidoglycan through ionic interactions (2,103) is dissociated in the presence of these high concentrations of NaCl. The insoluble peptidoglycan (along with covalently bound Braun's lipoprotein) was removed by centrifugation at 100,000 x g for 30 min. The solubilized porin was passed through Bio-Gel A-0.5M columns (Bio-Rad) equilibrated with 0.1 M sodium borate or sodium bicarbonate (pH 7.5), and 0.025% SDS. The elution of protein was monitored by its absorbance at 280 nm. The initial protein peak (Fig. 6.1) contained essentially pure porin (Fig. 6.2).

6.2.1.3 Coupling of Porin to Sepharose

Unless indicated, porin was coupled to Sepharose as follows. This procedure is essentially as outlined in the Pharmacia manual on affinity chromatography (104). The pH of the porin solution was adjusted to pH 9.0 with NaOH, and NaCl was added to 0.5 M. The protein was added to freshly prepared CNBr-activated Sepharose-4B, at a concentration of about 0.6-1 mg/ml of Sepharose. The mixture of porin and activated-Sepharose was mixed by end-over-end mixing for 3 hours at room temperature. The Sepharose was washed with water, and 0.2 M glycine (pH 8.0) was added to block unreacted groups. A control, without protein in the coupling buffer and blocked with glycine, (the so-called "glycine-Sepharose") was also prepared.

The glycine blocking was carried out overnight at 4°C, followed by washing with water, and alternating 0.1 M acetate, 0.5 M NaCl (pH 4.0), and 0.1 M borate, 0.5 M NaCl (pH 9.0) washes. Following the last borate wash, the gel was washed with water and then incubated for one hour with 1% bovine serum albumin (BSA) in 10 mM sodium phosphate, pH 6.6, to block any nonspecific protein binding sites. The gel was then treated

with 6 M guanidine-HCl, washed extremely well with phosphate, and kept at 4°C until used.

6.2.2 Porin-Sepharose Binding Assays in Microfuge Tubes

The binding of [^{35}S]-SEP to immobilized porin was easily assayed in microfuge tubes. The buffer was removed from porin-Sepharose and glycine-Sepharose by vacuum filtration. The Sepharose was weighed and 3 ml of 10 mM sodium phosphate was added per gram of gel. Buffer and [^{35}S]-SEP were added to 500 µl of gel suspension (final volume = 1.0 ml) in silane coated 1.5 ml microfuge tubes. The mixture was incubated for 10 min at room temperature with occasional inversion. The gel suspension was then spun for 2 min in a microcentrifuge and 700 µl of the supernatant was removed and counted (this is the unbound or "free" SEP). The difference between the total counts added and the free counts remaining is the amount of SEP "bound".

The "releasability" of the bound SEP was also determined to obtain an idea of the affinity of SEP for the immobilized porin. The Sepharose was washed two or three times with 1.0 ml of phosphate. 1.0 ml of "releasing agent" (for example 1 M NaCl or 6 M guanidine-HCl) was added and allowed to incubate for 20-30 min. After centrifugation a 1.0 ml aliquot was removed (the "releasable" fraction) and counted. The samples were added to 7-10 ml of Formula 947 (New England Nuclear) and the radioactivity counted in a Beckman Scintillation Counter.

If the porin-Sepharose was to be reused it was treated with 6 M guanidine-HCl to remove any remaining SEP, washed in phosphate, and stored overnight at 4°C before reuse. Guanidine-HCl was shown in preliminary experiments to remove SEP from the porin-Sepharose without substantially altering subsequent binding assays.

6.3 RESULTS

6.3.1 Purification of Porin

The elution profile of SDS-NaCl soluble material from the Bio-Gel A-0.5M column is shown in Fig. 6.1. The single early eluting peak contains porin. The purity of the porin is shown in Fig. 6.2. The SDS-PAGE gel shows porin isolated from JF701 ($OmpC^-$, $OmpF^+$) or JF703 ($OmpC^+$, $OmpF^-$). The porin was solubilized in sample buffer in the absence (-ME) or presence (+ME) of 2-mercaptoethanol. The porin appears pure and when nondenatured appears to be in a trimeric (i.e "native") form. The presence of multiple bands is indicative of the presence of tightly associated lipopolysaccharide (LPS) (98).

6.3.2 Optimization of Porin Coupling to Sepharose

[^{35}S]-labelled porin was isolated from M9 grown CBT43. Coupling of porin to CNBr-activated Sepharose was as outlined in Section 6.2.1.3 with the conditions listed below. Aliquots of Sepharose were counted to determine coupling efficiency.

6.3.2.1 Effect of Detergent Concentration

The effect of detergent (SDS or deoxycholate) concentration on the amount of [^{35}S]-porin coupled to CNBr-Sepharose was studied. These two detergents were chosen because they had been shown to be able to solubilize porin. As can be seen in Fig. 6.3A, increasing the SDS concentration decreased the amount of porin coupled by about 30% from 680 to 470 pmoles/100 mg of Sepharose. On the other hand, increasing the deoxycholate concentration caused a very significant increase in the amount of porin coupled. The maximum levels were reached between 0.5% and 1% deoxycholate. The amount of coupled porin increased from 500 to almost 1200 pmoles/100 mg (wet wt.) Sepharose, an increase of 130%.

FIGURE 6.1

BIO-GEL A-0.5M GEL FILTRATION OF PORIN

This figure presents the elution profile of NaCl-SDS releasable protein on a Bio-Gel A-0.5M gel filtration column. Outer membranes treated with SDS at 55°C, were then treated with 0.5 M NaCl, 2% SDS as described in Section 6.2.1.2. After centrifugation at 100,000 x g for 0.5 hr at 4°C, the supernatant was applied to a Bio-Gel A-0.5M column equilibrated with 0.025% SDS, 0.1 M sodium bicarbonate buffer (pH 7.5). The elution of the protein was followed at 280 nm and the elution of the salt peak was followed by conductivity. Porin is contained in the first peak (see Fig 6.1).

(●) - Absorbance at 280 nm.

(△) - Conductivity (mmho).

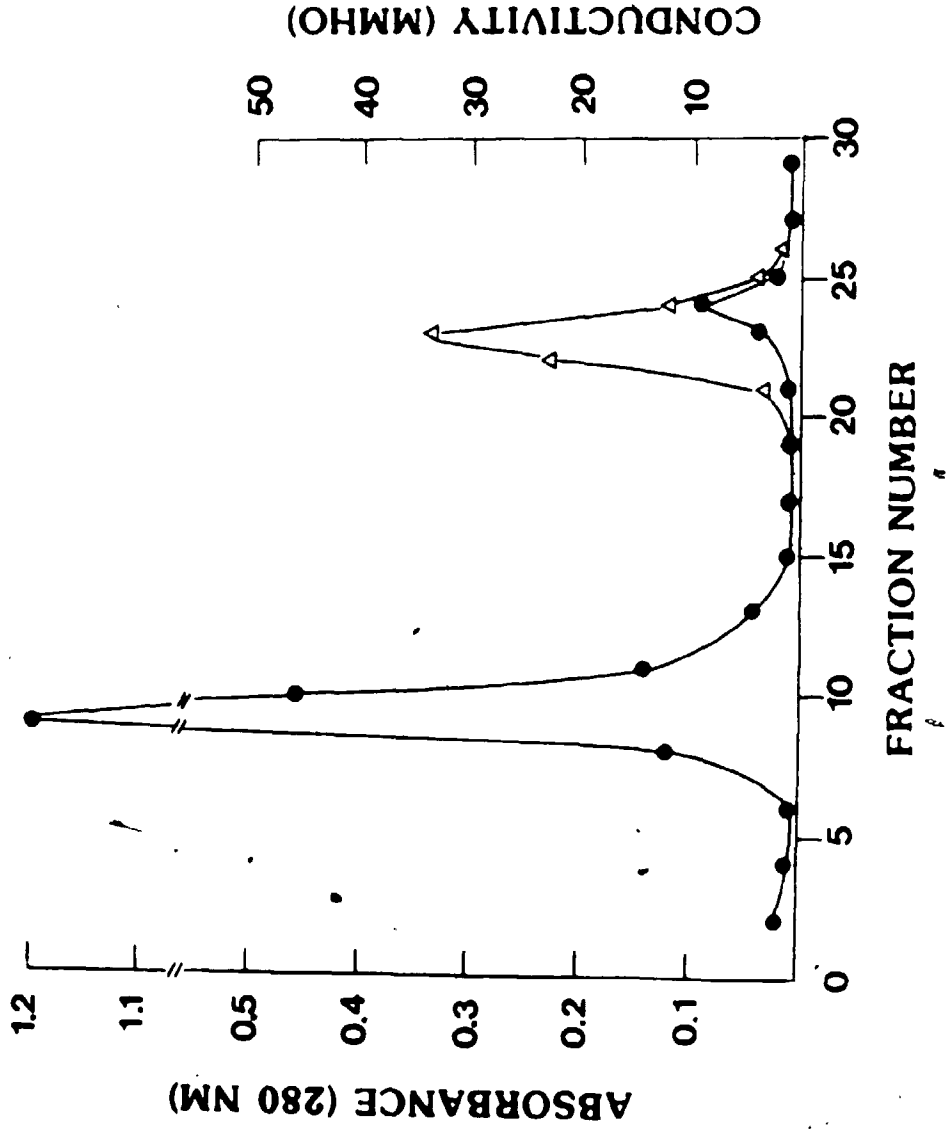
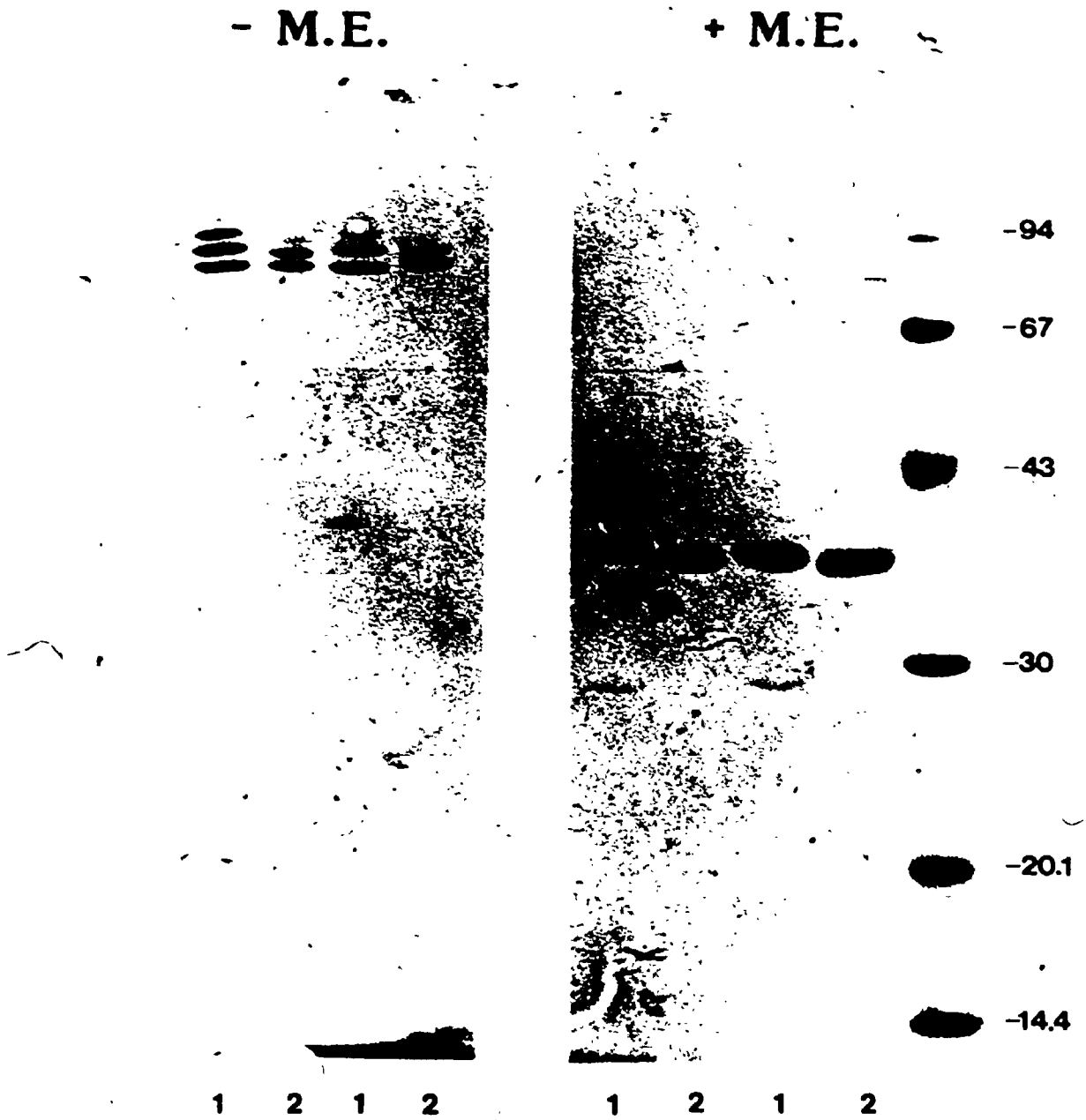

CONDUCTIVITY (MMHO)
50
40
30
20
10
30
25
20
15
10
5
0
FRACTION NUMBER
1.2
1.1
0.5
0.4
0.3
0.2
0.1
ABSORBANCE (280 NM)

FIGURE 6.2

PURITY OF THE ISOLATED PORIN

OmpF porin and OmpC porin isolated (as described in Fig. 6.1) from JF701 ($OmpC^-$, $OmpF^+$)(Lane 1) and JF703 ($OmpC^+$, $OmpF^-$) (Lane 2) respectively was run on discontinuous SDS-PAGE gels. Part of the sample was solubilized in the Laemmli sample buffer in the presence of 2-mercaptoethanol (5%) at 100°C (+ M.E). Porin was also solubilized in sample buffer without 2-mercaptoethanol at 37°C (- M.E.). The multiple banding seen in the absence of boiling and 2-mercaptoethanol is similar to that seen by Gavasito et al (98) and is believed to be indicative of the presence of associated LPS. The high molecular weight is indicative of native trimeric structure.


- M.E.
+ M.E.
-94
-67
-43
-30
-20.1
-14.4
1 2 1 2
1 2 1 2

This corresponds to 70% of the porin added.

6.3.2.2 The Effect of pH

The effect of pH of the coupling buffer on the coupling efficiency was also investigated. This was done using 0.025% SDS or deoxycholate in sodium bicarbonate buffer. The pH was adjusted with NaOH or HCl.

It can be seen from Fig. 6.3B that as the pH of the coupling reaction increases, the amount of porin coupled to Sepharose increases in both deoxycholate and SDS. The increase was more substantial for deoxycholate, a 186% increase, compared to an 80% increase for SDS.

6.3.2.3 Stability of the Coupled Porin

In order to determine if the "coupled" porin was attached to the Sepharose in a stable manner, [^{35}S]-porin-Sepharose prepared at the various detergent concentrations and pH values was treated with 1% Lubrol; 1% SDS; 1 M NaCl; or 6 M Guanidine-HCl.

From Figs. 6.3C and 6.3D it can be seen that 6 M Guanidine-HCl released less than 6% of the counts from the porin-Sepharose prepared at various detergent concentrations, and NaCl (not shown) had no effect. On the other hand Lubrol and SDS released a significant percentage of the radioactivity. The percentage of counts released was greatest when the coupling was performed at low detergent concentrations. In the case of deoxycholate coupled porin, this was also where coupling efficiency was least. Therefore, as the deoxycholate concentration increased, coupling became more efficient and nonspecific hydrophobic interactions was reduced. It was shown above that coupling efficiency seemed to decrease slightly with increasing SDS concentrations, but as can be seen here, this decrease may be due to a reduction of noncovalent hydrophobic interactions.

FIGURE 6.3

OPTIMIZATION OF PORIN COUPLING TO SEPHAROSE

[^{35}S]-porin was equilibrated with 0.025% SDS or 0.025% deoxycholate by passage through BioGel A-0.5M equilibrated with the detergents in 0.1 M sodium bicarbonate, pH 8.5, 0.5 M NaCl. The protein was coupled to CNBr-Sepharose, blocked with glycine, subjected to alternate pH washing (in 0.5 M NaCl), resuspended in phosphate buffer and aliquots were counted. 0.63 mg of porin per gram of Sepharose was added (equivalent to 1700 pmoles/100 mg Sepharose).

(A) Effect of detergent concentration.

Detergent (SDS or deoxycholate) was added to give final concentrations of up to 2% in the coupling reaction (pH 8.5).

SDS (△); deoxycholate (●)

(B) Effect of pH on coupling.

The pH of the solution containing the eluted protein (with 0.025% SDS or 0.025% deoxycholate) was adjusted with HCl or NaOH before coupling.

SDS (△); deoxycholate (●).

(C-F) Stability of coupled porin.

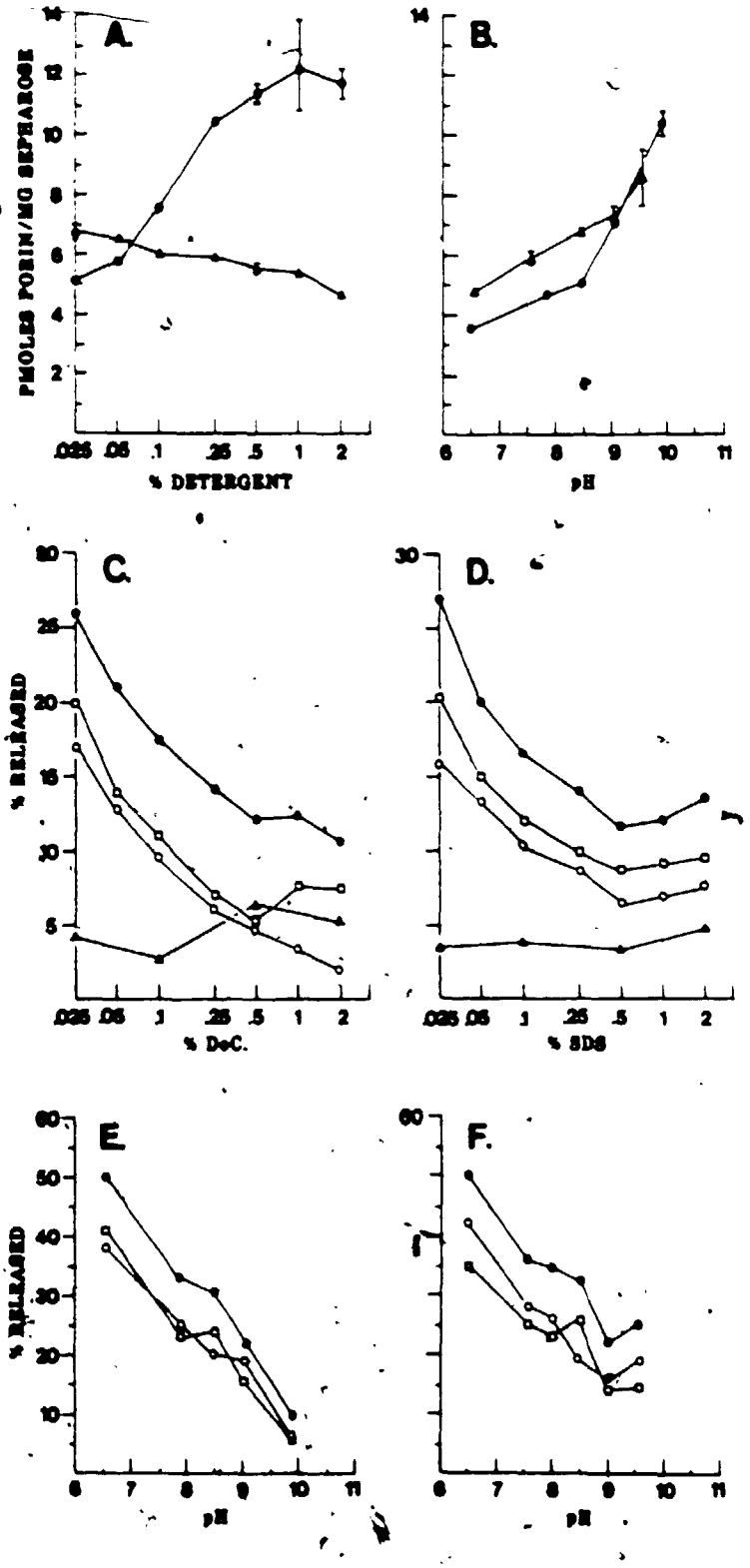
The [^{35}S]-porin Sepharose coupled under the various conditions was then treated with various agents to determine if the porin was covalently coupled. The protein released is expressed as a percentage of that originally coupled. Releasing agents include 6 M guanidine-HCl (▲); 1% SDS (○); 1% Lubrol (□); or sequential 1% SDS and 1% Lubrol (●).

(C) Release of porin from porin-Sepharose prepared using various deoxycholate concentrations.

(D) Release of porin from porin-Sepharose prepared using various SDS concentrations.

(E) Release of porin from porin-Sepharose prepared in 0.025% deoxycholate at various pH values.

(F) Release of porin from porin-Sepharose prepared in 0.025% SDS at various pH values.


A.
B.
C.
D.
E.
F.
PMOLES PORIN/MG SEPHAROSE
% DETERGENT
pH
% RELEASED
% DoC.
% SDS
.025 .05 .1 .25 .5 1 2
6 7 8 9 10 11

At low pH values, up to 50% of the radioactivity was associated with the Sepharose through noncovalent interactions (Figs. 6.3E and 6.3F). Again this was at a pH at which coupling efficiency was lower in both SDS and deoxycholate.

6.3.3 Binding of SEP to Columns of Porin-Sepharose

Initial in vitro porin-Sepharose binding assays were carried out in small columns (<2 ml). [^{35}S]-labelled SEP was added and the column was washed with 10 mM sodium phosphate (pH 7) buffer. Material bound to the column was eluted with 1 M NaCl. The purity of the radioactive SEP was shown in Chapter 2. Both the EDTA-sucrose and osmotic shock SEP used in this study consisted of a single labelled species with a molecular weight of 56K. The SEP had been stored frozen at -20°C. The protein retained its ability to bind to aspartate-Sepharose (Fig. 6.4D). This indicated that freeze-thawing had not significantly denatured the SEP.

Fig. 6.4 shows the elution profile of EDTA-sucrose SEP (Fig. 6.4A) or osmotic-shock SEP (Fig 6.4B) on the porin-Sepharose columns. It is obvious that the SEP bound tightly to the porin-Sepharose. The radioactivity applied to the porin-Sepharose was not fully recovered. Typically 65-70% of the counts were recovered.

The proteins that did not bind to the aspartate-Sepharose columns were also applied to the porin-Sepharose columns. Fig. 6.4C shows the elution profile of these non-retained proteins. Only a percentage of this protein bound to the porin-Sepharose. This can be used as an indication of the specificity of the porin-Sepharose.

Considerable nonspecific binding of SEP was seen with the glycine-Sepharose control (Fig. 6.4E) although SEP did not bind to unmodified Sepharose (Fig 6.4F).

FIGURE 6.4

BINDING OF [^{35}S]-SEP TO COLUMNS OF PORIN-SEPHAROSE

Initial SEP:porin-Sepharose binding assays were carried out in small columns of porin-Sepharose. [^{35}S]-labelled SEP was added and the column was washed with 10 mM sodium phosphate (pH 7.0) to remove non-bound protein. Protein bound to the column was eluted with 1 M NaCl. The column elution profiles were obtained by counting the radioactivity of collected fractions. SEP derived from EDTA-sucrose (A) and osmotic shock (B) as well as osmotic shock releasable proteins not retained by aspartate-Sepharose (C) were all passed through porin-Sepharose.

Control columns included; Aspartate-Sepharose (D), to ensure SEP still retained its ability to bind to the affinity matrix; "Glycine-Sepharose" (E) prepared similarly to porin-Sepharose but without protein in the coupling buffer; and untreated Sepharose-4B (F).

(A-C) Porin-Sepharose columns of:

(A) EDTA-sucrose SEP.

(B) Osmotic shock SEP.

(C) Osmotic shock releasable proteins not retained by aspartate-Sepharose ("Aspartate-Sepharose Pass-through").

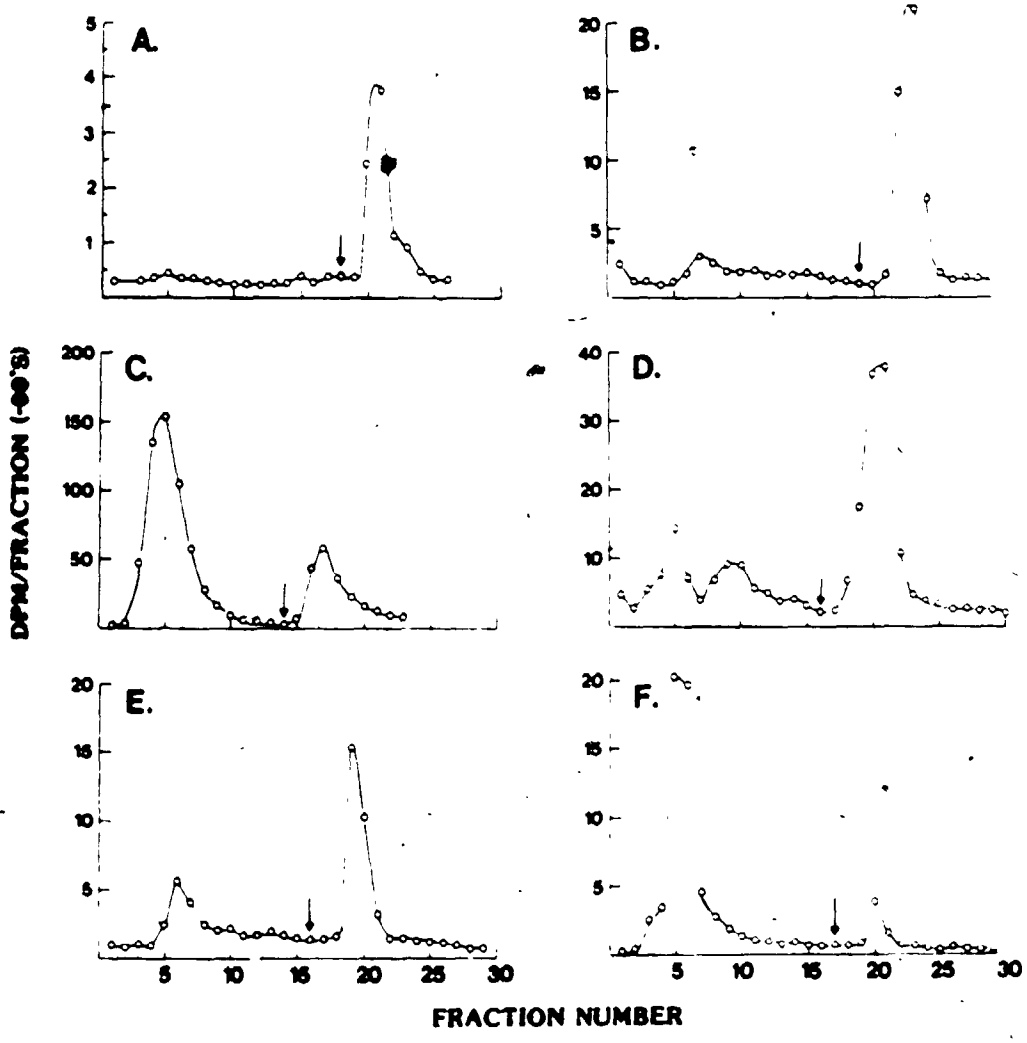
(D-F) Control columns:

(D) Osmotic shock SEP on aspartate-Sepharose.

(E) Osmotic shock SEP on glycine-Sepharose.

(F) Osmotic shock SEP on un-modified Sepharose-4B.

The arrows indicate where 1 M NaCl was added.


A.
B.
C.
D.
E.
F.
DPM/FRACTION (-●●'S)
FRACTION NUMBER

Both EDTA-sucrose and osmotic shock SEP gave similar results. Given the large amount of SEP that could be isolated from osmotic shock, this population of the protein was used in the majority of later studies and will be referred to as SEP.

6.3.4 Microfuge Tube Binding Assays

The binding of SEP to porin-Sepharose was more easily assayed in microfuge tubes than on columns. These assays consisted of mixing the porin-Sepharose and SEP in a microfuge tube, allowing binding to occur, and then centrifuging to pellet the porin-Sepharose. The supernatant was counted to determine the amount of free SEP remaining, and by difference the amount of SEP bound was calculated.

6.3.4.1 Effect of SEP Concentration in Binding Assay

Various amounts of SEP were added to porin-Sepharose and glycine-Sepharose (Fig. 6.5). It can be seen that increasing the amount of SEP results in a saturation of the glycine-Sepharose binding, so that the difference between porin- and glycine-Sepharose becomes more apparent at higher concentrations of SEP.

6.3.4.2 Effect of SDS in Coupling Buffer

The large amount of nonspecific SEP binding to glycine-Sepharose may have been due to the presence of detergent in the coupling buffer. Various concentrations of SDS were added to the coupling buffer (pH 9.0) in the preparation of glycine-Sepharose, and SEP binding was assayed.

As can be seen in Fig. 6.6, the most significant change was the increase in nonspecific binding between 0 and 0.0032% SDS. The presence of small quantities of SDS in the coupling buffer appears to create nonspecific and non-NaCl releasable SEP binding sites. There was a slight decline in the nonspecific "total" binding of SEP to the glycine-

FIGURE 6.5

EFFECT OF SEP CONCENTRATION ON BINDING TO PORIN-SEPHAROSE AND GLYCINE-SEPHAROSE

Various concentrations of SEP were added to porin-Sepharose and glycine-Sepharose in microfuge tube binding assays. The amount of [^{35}S]-SEP bound was obtained as the difference between the counts added and the free counts remaining after centrifugation.

(●) - SEP bound to porin-Sepharose

(□) - SEP bound to glycine-Sepharose

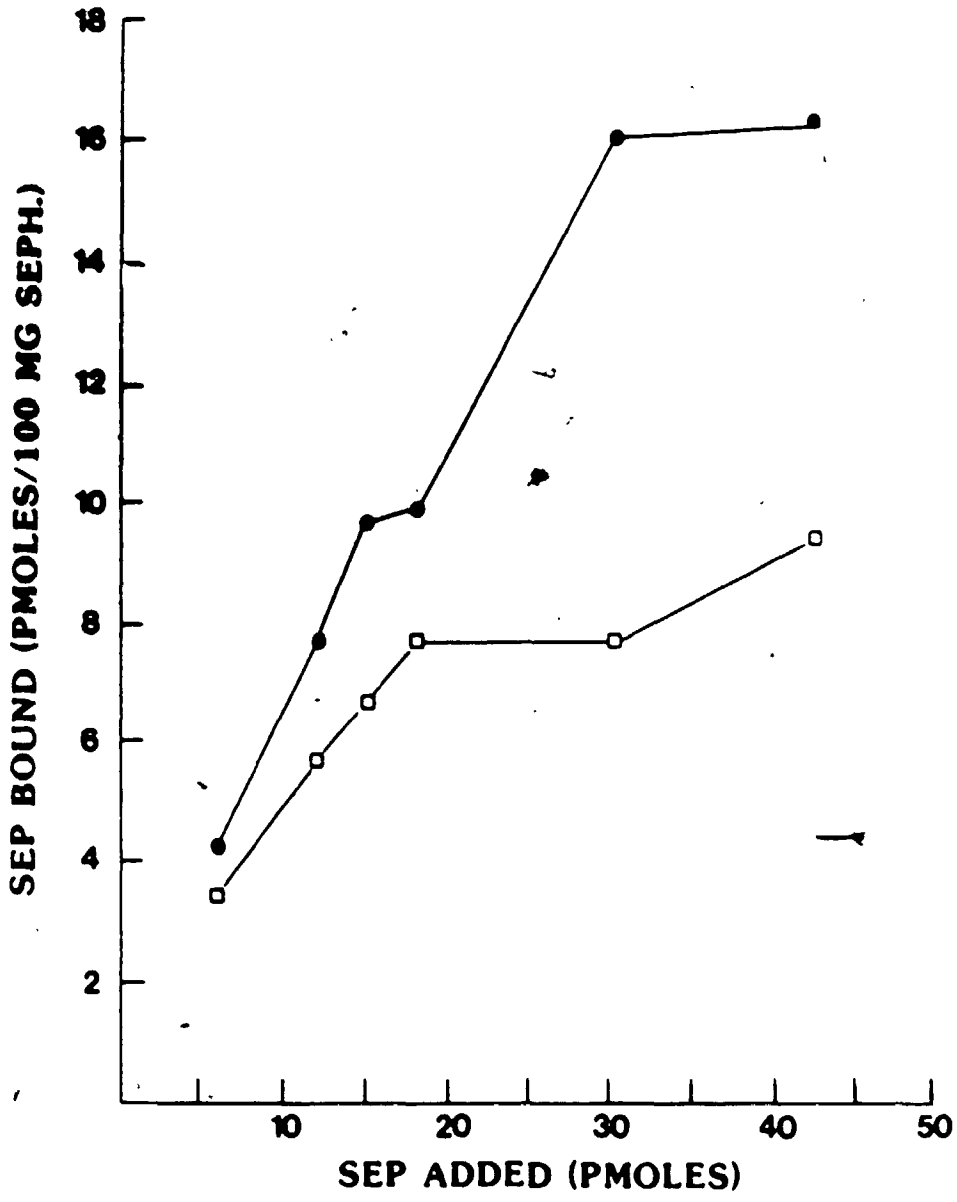

SEP BOUND (PMOLES/100 MG SEPH.)
2
4
6
8
10
12
14
16
10
20
30
40
50
SEP ADDED (PMOLES)

FIGURE 6.6.

EFFECT OF SDS IN COUPLING BUFFER ON [^{35}S]-SEP BINDING TO GLYCINE-SEPHAROSE

To determine if detergent in the coupling buffer has an effect on the amount of SEP that binds nonspecifically to Sepharose, various concentrations of SDS were added to the coupling buffer (pH 9.0) in the preparation of glycine-Sepharose. Binding of [^{35}S]-SEP to the glycine-Sepharose was assayed in microfuge tubes as outlined in "Experimental Procedures". 39 pmoles/100 mg of Sepharose was added. The amount of SEP bound to the Sepharose (●) and the amount released by 1 M NaCl (○) was determined.

FIGURE 6.7.

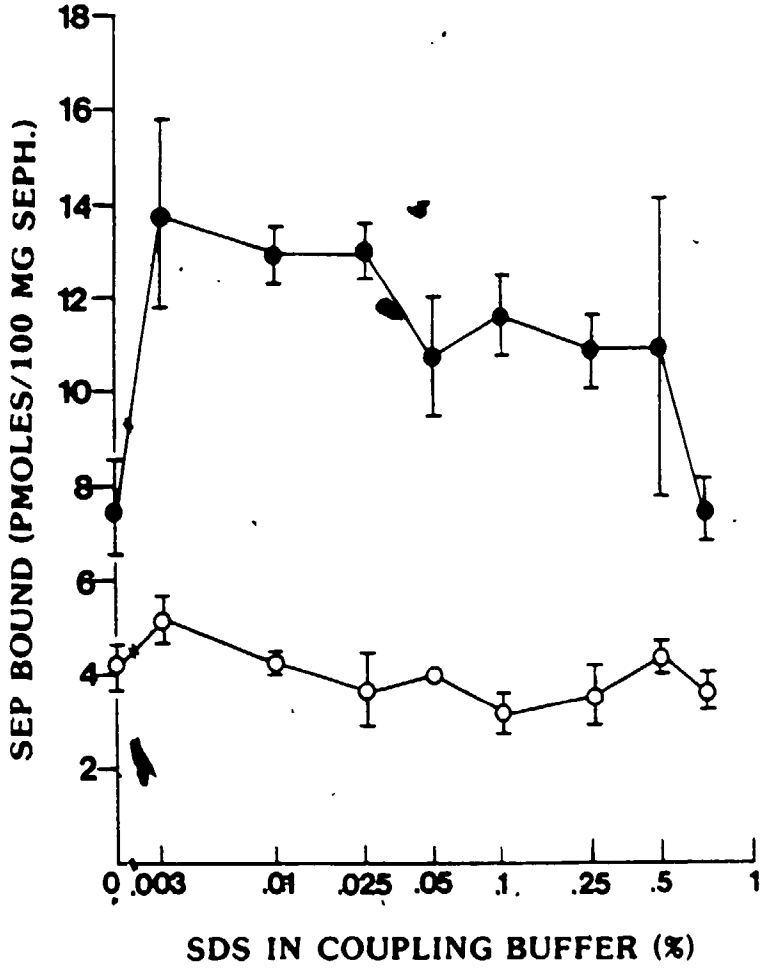
EFFECT OF SDS ON [^{35}S]-SEP BINDING TO MICROFUGE TUBES

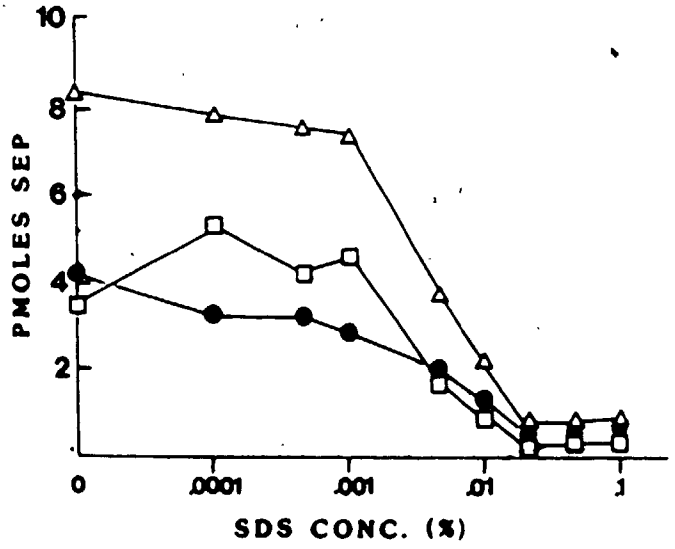
Purified SEP, in 10 mM phosphate buffer (pH 7.0) at a concentration equal to that used in the binding assay, was incubated in empty microfuge tubes for 35 min at room temperature in the presence of low concentrations of SDS (0 - 0.1%). The tubes were washed with phosphate and treated with 6 M guanidine-HCl. Protein released by guanidine-HCl and remaining with the tubes was counted.

(□) - Guanidine-HCl releasable

(●) - Remaining with the tube

(△) - Total = (releasable + tube)


SEP BOUND (PMOLES/100 MG SEPH.)
18
16
14
12
10
8
6
4
2
0
.003
.01
.025
.05
.1
.25
.5
1
SDS IN COUPLING BUFFER (%)


PMOLES SEP
10
8
6
4
2
0
.0001
.001
.01
.1
SDS CONC. (%)

Sepharose as the concentration of SDS in the coupling buffer increased from 0.0032% to 0.5%. The counts that were releasable by 2 M NaCl did not change very significantly over this same range.

6.3.4.3 Nonspecific Interaction with Microfuge Tubes

During the course of the binding studies it became obvious that SEP bound nonspecifically to the microfuge tubes. To determine the extent of binding to the microfuge tubes, binding assays were carried out in the absence of Sepharose. Treating the tubes with silane decreased binding by about 20–40%. The effect of SDS on binding to the tubes was also investigated (Fig. 6.7). It is clear that above 0.01% SDS, the nonspecific binding decreases substantially, and reaches a minimum at 0.025% SDS.

6.3.4.4 Effect of SDS on SEP Binding to Porin-Sepharose

As SDS was shown to reduce nonspecific binding to microfuge tubes in a very significant manner, we felt it would be advantageous to see if these low concentrations of SDS would decrease the amount of nonspecific binding to Sepharose.

As can be seen in Fig. 6.8, at low SDS concentrations there was both a decrease in binding to the glycine–Sepharose and a very significant increase in binding to porin–Sepharose. When determined from either the free counts remaining in solution (Fig. 6.8A), or from counts releasable by 6 M guanidine–HCl (Fig. 6.8B), there was a very substantial increase in the apparent "specificity" of binding to porin-Sepharose as compared to glycine–Sepharose.

The effect of increasing the ionic strength on SEP binding in the presence or absence of 0.01% SDS was investigated (Fig. 6.9). Whereas low concentrations of NaCl (10–50 mM) caused a substantial reduction in

FIGURE 6.8.

EFFECT OF SDS ON [^{35}S]-SEP BINDING TO PORIN-SEPHAROSE AND GLYCINE-SEPHAROSE

[^{35}S]-SEP was incubated with porin-Sepharose and glycine-Sepharose in 10 mM sodium phosphate pH 7.0 in the presence of low concentrations of SDS (0 - 0.05%). Total SEP bound to the Sepharose was determined as the difference between the total counts added and the free counts remaining. The Sepharose was treated with 6 M guanidine-HCl and the releasable SEP was determined.

(A) Total SEP bound

(B) 6 M guanidine-HCl releasable SEP

(●) - SEP bound to porin-Sepharose

(□) - SEP bound to glycine-Sepharose

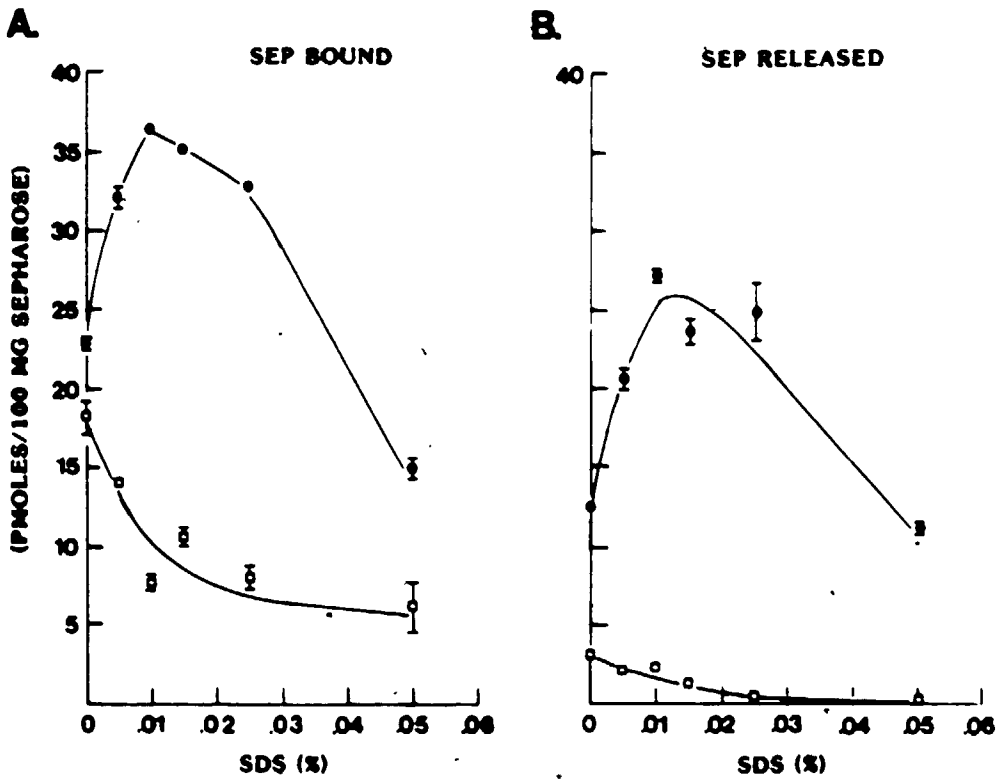
FIGURE 6.9.

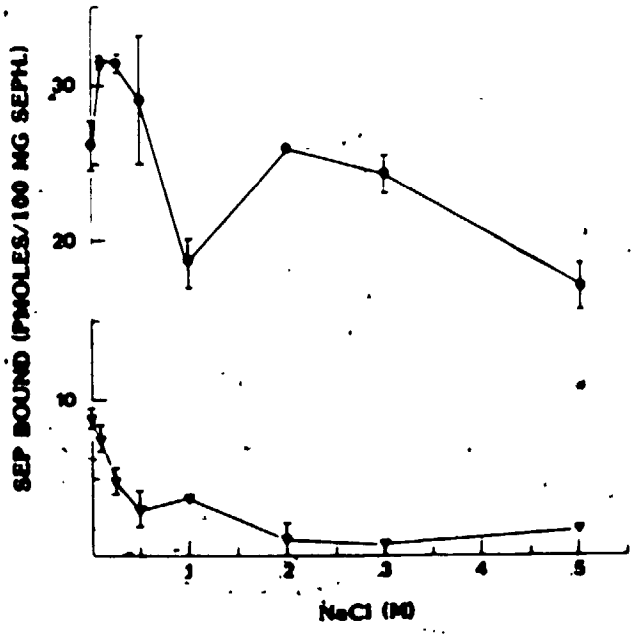
EFFECT OF NACL ON BINDING OF SEP TO PORIN-SEPHAROSE IN THE PRESENCE OF SDS

[^{35}S]-SEP was incubated with porin-Sepharose and glycine-Sepharose in the presence or absence of 0.01% SDS and NaCl concentrations ranging from 0 - 0.5 M. The amount of SEP specifically bound to porin-Sepharose was determined as the difference between the counts bound to porin-Sepharose and glycine-Sepharose.

(●) - SEP bound in the presence of 0.01% SDS

(▽) - SEP bound in the absence of SDS


A.
SEP BOUND
B.
SEP RELEASED
(PMOLES/100 MG SEPHAROSE)
40
35
30
25
20
15
10
5
0
.01
.02
.03
.04
.05
.06
SDS (%)


SEP BOUND (PMOLES/100 MG SEPH.)
20
10
1
2
3
4
5
NaCl (M)

SEP binding in the absence of SDS, these same concentrations caused an increase in specific binding in the presence of SDS. Considerable specific interaction was seen even in the presence of 0.5 M NaCl when SDS was present.

6.3.4.5 Effect of Other Detergents and LPS on SEP Binding

Low concentrations (below the critical micelle concentration) of nonionic detergents (Triton and Lubrol) and a wide concentration range of LPS were tested for their effect on the SEP:porin-Sepharose binding. As can be seen in Table 6.1, there was no dramatic increase in binding to porin-Sepharose as had been seen with SDS.

6.3.4.6 Effect of Succinate on SEP Binding

Various concentrations of succinate were tested for their effect on SEP binding in the absence of detergents. It can be seen (Table 6.2) that this substrate for the dicarboxylic acid transport system had no noticeable effect on binding.

6.3.5 Binding to Porin-Sepharose from Different Bacterial Strains

To determine if SEP bound *in vitro* to different species of porin (OmpC, OmpF, PhoE) with equal affinity, porin-Sepharose was prepared from porin isolated from the isogenic porin mutants JF568(parental, $OmpC^+$, $OmpF^+$), JF694($PhoE^+$, $OmpC^-$, $OmpF^-$), JF701($OmpC^-$, $OmpF^+$), JF703($OmpC^+$, $OmpF^-$). These strains were grown in [^{35}S]-supplemented M9 to label the porin. The isolation and coupling were carried out under the optimal conditions determined for CBT43.

Equal volumes of porin-Sepharose were used in the binding assays, but due to differences in the coupling, the amount of porin present (as determined from radioactivity) was different from strain to strain.

As can be seen from Table 6.3, the amount of SEP bound and the

TABLE 6.1

EFFECT OF DETERGENTS AND LPS ON SEP BINDING TO PORIN-SEPHAROSE

ADDITION	CONCENTRATION	SEP BOUND (pmoles/100 mg Sepharose)	PERCENT OF CONTROL
CONTROL		8.8	100
TRITON X-100 (Amersham)	0.0005%	11.3	129
	0.0001	10.9	124
	0.005	10.0	114
LUBROL (17A10, ICI)	0.0005%	12.5	142
	0.0001%	10.9	124
	0.005%	9.7	110
LPS (*E. coli* 026:86 Phenol extract, Sigma)	0.5 ug/ml	11.5	131
	5 ug/ml	7.6	86
	20 ug/ml	7.2	82
	50 ug/ml	8.7	98

Binding of SEP to porin-Sepharose and glycine-Sepharose was carried out in the presence of various concentrations of detergents and LPS. The amount of SEP specifically bound to porin-Sepharose was determined. 39.5 pmoles of SEP per 100 mg of Sepharose was added.

TABLE 6.2

EFFECT OF SUCCINATE ON SEP BINDING TO PORIN-SEPHAROSE

SUCCINATE (uM)	SEP BOUND (pmoles/100 mg Seph.)	PERCENT OF CONTROL
0	9.8 ± .5	100 ± 5
20	9.05 ± 2	92 ± 22
50	9.14 ± .6	93 ± 6
100	9.04 ± .3	92 ± 3

Various concentrations of succinate were added to the SEP:Sepharose binding assay (in the absence of detergent) and the amount of SEP specifically bound to porin-Sepharose was determined. 40 pmoles SEP per 100 mg/Sepharose was added.

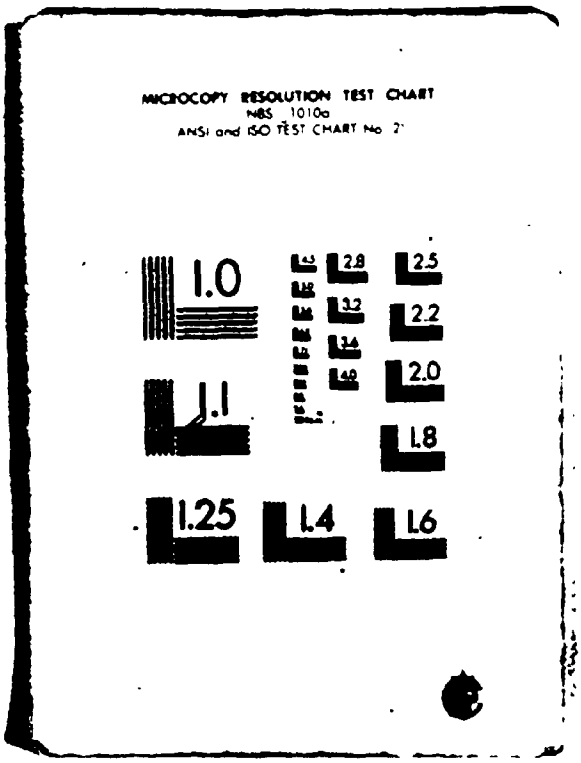

MICROCOPY RESOLUTION TEST CHART
NBS 1010a
ANSI and ISO TEST CHART No 2
1.0
1.1
1.25
1.4
1.6
2.8
3.2
3.6
2.5
2.2
2.0
1.8

TABLE 6.3

BINDING OF SEP TO PORIN-SEPHAROSE PREPARED FROM VARIOUS STRAINS

STRAIN	PORIN COUPLED (pmoles/100 mg Seph.)	SEP BOUND (pmoles/100 mg Seph.)	OCCUPANCY (%)
JF568 (parental, $OmpC^+$, $OmpF^+$)	370	56.1	15
JF694 ($OmpC^-$, $OmpF^-$, $PhoE^+$)	440	57	13
JF701 ($OmpC^-$, $OmpF^+$)	434	56	13
JF703 ($OmpC^+$, $OmpF^-$)	910	56	6.2

To determine if SEP bound *in vitro* to different species of porin (OmpC, OmpF, PhoE) with equal affinity, porin-Sepharose was prepared from porin isolated from the isogenic porin mutants JF568 (parental, mostly OmpC), JF694 (PhoE), JF701 (OmpF), and JF703(OmpC). These strains were grown in [^{35}S]-supplemented M9 to label the porin. The isolation and coupling were carried out under the optimal conditions determined for CBT43 (0.5% deoxycholate, pH 9). Before use the porin-Sepharose was blocked with 0.4% BSA, and washed with 6 M guanidine-HCl and 1% SDS, followed by extensive phosphate buffer washing on a scintered glass funnel. Equal volumes of porin-Sepharose were used in the binding assays. 81 pmoles of SEP per 100 mg of Sepharose was added. The "Occupancy" is the percentage of porin with SEP bound to it, calculated as 1 molecule of SEP per molecule of porin.

counts released were almost identical from strain to strain. This finding is in agreement with the whole cell binding studies in which little strain to strain variation was seen. The "occupancy" varied from 6% of available porin in the case of JF703 to 15% of available porin for JF568 (in terms of binding of 1 SEP to 1 molecule of porin). The porin isolated from the parental JF568 is primarily OmpC under the growth conditions used. The reason for the differences in amount of porin coupled (910 pmole/100 mg of gel for JF703 as compared to 370-440 pmole/100 mg for the other strains) is not clear as similar proteins are being coupled. Perhaps the JF703 porin preparation was contaminated with some other radioactive component such as sulfolipids.

In each case the amount of SEP bound corresponds to 75% of that applied. This may be the maximal amount available for binding. The low occupancy of the sites, allowing maximal binding, may mask any difference between the strains.

6.4 DISCUSSION

The experiments presented in Chapter 5 indicated that exogenously added purified SEP could interact with whole cells, and we decided to investigate this further, using an *in vitro* system with porin coupled to an inert matrix of Sepharose. The porin used for the coupling was free of other contaminating proteins, and was in an apparently native conformation being in the trimeric form and containing tightly associated LPS. A native conformation is essential in order to relate these *in vitro* studies to the *in vivo* situation.

The coupling of porin to CNBr-activated Sepharose was optimized. Porin coupled under optimal conditions was less likely to be removed by

subsequent treatment of the porin-Sepharose with releasing agents such as guanidine-HCl.

Initial experiments indicated that SEP isolated from aspartate-Sepharose bound to porin-Sepharose but not to unmodified Sepharose. Similar elution patterns were obtained for SEP isolated by osmotic shocking or by EDTA-sucrose treatment. The interaction with the columns seemed to be quite strong. A large percentage of the protein could be eluted with 1 M NaCl, but a fairly substantial percentage could not be dissociated from the Sepharose. A protein-free control of "glycine-Sepharose" (made in the same manner as the porin-Sepharose but without protein) also bound a large percentage of the SEP, indicating considerable nonspecific interaction was taking place.

Low concentrations of SDS were shown to reduce nonspecific interactions between SEP and glycine-Sepharose, while at the same time increasing the amount of SEP bound to porin-Sepharose. A similar effect was not seen with the nonionic detergents Lubrol or Triton X-100, or with bacterial lipopolysaccharide. The SDS effect was only seen within a very narrow concentration range. Although a range of concentrations were used with LPS and the other detergents it is possible that a similar optimal point was missed. Slight increases in ionic strength (i.e up to 50 mM) caused an increase in SEP binding in the presence of SDS but a reduction in SEP binding in its absence. This indicates that the interaction with Sepharose is largely ionic in the absence of SDS but hydrophobic in its presence.

It can be seen from many of the studies that SDS had an effect on numerous aspects of the *in vitro* reaction. What is the detergent affecting? As we are looking at a protein-protein interaction, the

conformation of the proteins is of great importance. Since one of the components (porin) is an integral membrane protein it is likely that lipids or lipopolysaccharide are important in maintaining its conformation. In the absence of lipids, detergents may substitute. This may be what is occurring at low concentrations of SDS. The detergent may be restoring native conformation to the bound porin, increasing its ability to bind SEP. At the same time, the blockage of nonspecific binding sites (on the Sepharose and microfuge tubes), and possible prevention of aggregation, will increase the amount of SEP available to bind. A less attractive hypothesis for the action of SDS is that this anionic detergent is binding to the porin by its hydrophobic moiety and cross linking it to SEP through its negatively charged end.

Nonionic detergents such at Triton or Lubrol did not give rise to a similar specific increase in binding to porin-Sepharose. These detergents are unable to solubilize outer membrane components *in vivo*, and thus may be unable to mimic the native porin microenvironment. On the other hand lipopolysaccharide which is found naturally in the outer membrane (and seems to be required for binding to whole cells (Chapter 5)) was unable to substitute for SDS *in vitro*. Perhaps the concentration was incorrect or the method of presentation was unsuitable. LPS is often used in studies where porin is reconstituted into vesicles or black-lipid films, but these systems offer a lipid phase in which the lipid-A portion could presumably obtain a natural conformation. The lack of a lipid phase in the porin-Sepharose studies might preclude the lipid-A from attaining this conformation and hence LPS-dependent SEP binding was not seen. The SDS-PAGE profiles indicate

that the purified porin contains tightly associated LPS, although we have not tested this chemically. Perhaps this level of LPS is sufficient for SEP binding (in the presence of SDS to restore conformation) and added LPS would simply be an excess.

High concentrations of SDS were shown to abolish the porin-Sepharose : SEP interaction. In this case the SDS may be completely covering either the porin or SEP, thus preventing binding or causing denaturation of one or both of the components.

Use of porin-Sepharose prepared using different species of porin, showed little variation in the amount of SEP bound. A constant percentage of the added SEP was bound, indicating that the SEP could not distinguish between the species of porin under the artificial in vitro conditions, a result similar to that seen in the whole cell binding studies.

In summary, we have shown that SEP can bind specifically to immobilized porin, with maximum specificity being seen in the presence of low concentrations of SDS. In the presence of these low concentrations of SDS significant binding was seen. Although the nature of this in vitro reaction and how it compares to the binding with whole cells is far from clear, this in vitro study does indicate SEP can interact with various porin species, as demonstrated by whole cell studies.

CHAPTER 7

SUMMARY AND GENERAL DISCUSSION

7.1 SUMMARY OF RESULTS

The research presented in this thesis was undertaken to examine the properties and possible role of a major cell envelope protein in cell surface functions, such as dicarboxylate transport. This protein (SEP) was found to be one of the major proteins released by osmotic shock treatment. This suggests that SEP may be a major component of the cell envelope. This protein was isolated by aspartate-Sepharose affinity chromatography using conditions similar to those used by Bewick (46,58) (i.e. in 10 mM phosphate) for the isolation of DBP, with the addition of protease inhibitors phenylmethylsulfonyl fluoride (PMSF) and EDTA. The columns were originally eluted with 200 mM succinate and a protein with a molecular weight of 56K was eluted. This molecular weight differed significantly from the molecular weight of 16K reported by Lo and Sanwal (44) for DBP and so the protein isolated here was referred to as the succinate eluted protein (SEP). When [^{35}S]-labelled SEP was analysed, a single radioactive protein species was seen on SDS-PAGE gels, on IEF gels, and by HPLC/gel filtration. A similar protein was isolated from both the osmotic shock fluid and the EDTA-sucrose releasable fraction. SEP was shown to stimulate succinate uptake by whole cells when added externally.

Coomassie blue stained SDS-PAGE gels with increased sample loads indicated the presence of several contaminating proteins of lower molecular weight along with the major 56K protein. To further purify

SEP, conditions for growth and isolation were optimized. Since SEP was assumed to play a role in dicarboxylate transport, growth conditions were chosen for maximal succinate transport activity. Growth in minimal media (M9) was found to provide both maximal uptake and increased amounts of SEP in the osmotic shock fluid, suggesting a correlation between uptake and SEP levels. In order to establish the location of SEP in the cell envelope, conditions for osmotic shocking were selected in which release of cytoplasmic components was minimal. Cytoplasmic markers included DNA, RNA, and β-galactosidase. Cell envelope proteins from M9-grown cells also had a distinctive pattern on SDS-PAGE gels, with the 56K SEP and a 52K protein being major protein species. Contamination by cytoplasmic proteins was not visible on these gels using the optimized shocking conditions.

Conditions for aspartate-Sepharose affinity chromatography were manipulated to allow maximal recovery of SEP with minimal contamination. It was found that lower pH values (pH 6.6) were optimal for SEP retention by the column. The interaction of SEP with the aspartate-Sepharose was not that strong as SEP could be eluted with 30 mM phosphate buffer. The "contaminating" proteins were still bound to the column under these elution conditions.

SEP was characterized by physicochemical means. SEP had an isoelectric point of about 6.2. HPLC/gel filtration of the SEP indicated a native molecular weight close to ovalbumin (43K). An unusual behaviour of the protein was noted on SDS-PAGE in which, in the absence of 2-mercaptoethanol, migration was markedly slower than expected. Antibodies made against the oligopeptide binding protein from *E. coli* W (71), cross-reacted with SEP. Presently available data did

not allow us to determine whether these two proteins can carry out similar functions or if they are only similar in their physicochemical properties.

SEP was shown to stimulate whole cell succinate uptake (at 2 μM succinate) when added exogenously. Several approaches were used to determine its role in dicarboxylate transport. These approaches included (i) the interaction of SEP with succinate (ii) the interaction of SEP with cell surface components (iii) the interaction of SEP with immobilized porin and (iv) the correlation between the levels of SEP and transport activity.

Attempts made to demonstrate succinate binding to SEP were without success. Various succinate transport mutants were screened, but changes in transport activity could not be correlated with changes in SEP levels. These studies show that SEP does not possess a binding site for succinate and it is not coded by genes responsible for DBP or the two cytoplasmic membrane components. There was, however, some correlation in wild-type cells between the amount of SEP present and succinate uptake activity when cells were grown on rich medium or minimal (M9) medium.

The ability of SEP to stimulate succinate uptake when added to whole cells, indicated that SEP may be involved, directly or indirectly, in succinate transport. To investigate the interaction with the cell surface [^{35}S]-labelled SEP was added to whole cells. The SEP was shown to bind very tightly with the cells (K_D= 30 nM). The presence of porin and LPS was required, indicating a degree of specificity. An interesting observation was that *E. coli* mutants with defective DBP, were found to bind more SEP than the parental strain. In other types

of dicarboxylate transport mutants a correlation existed between the ability to bind SEP and the ability to transport succinate. All of the mutants that were unable to bind SEP were also defective in succinate transport. These mutant studies strongly suggest the involvement of SEP in dicarboxylate transport and that the interaction of SEP with the cell surface is essential for the transport process.

An artificial system was also used to study SEP binding to porin. This system consisted of porin coupled to CNBr-activated Sepharose. Low concentrations of SDS were shown to both decrease nonspecific binding as well as increase binding to porin-Sepharose. The interpretation of the action of low concentrations of SDS is not clear but it may be restoring a more natural conformation to the coupled porin while, at the same time, blocking nonspecific sites. This study also serves to demonstrate that SEP binds specifically to porin, as shown to be the case with whole cells. There was no difference in SEP binding to different species of porin isolated from various porin mutants. This is again in agreement with the whole cell studies.

7.2 DISCUSSION AND FUTURE DIRECTIONS

This study raises a number of questions that could be investigated further. A major question arises as to how a protein can stimulate succinate uptake when, in its pure form, it is apparently unable to bind succinate? Because of the affinity of SEP for porin it is possible that our inability to measure binding activity may be due to a requirement for the porin-SEP interaction. Succinate binding was not measured in the presence of porin or LPS. It is possible that formation of an SEP-porin complex induces a conformational change in SEP which then promotes

binding. The earlier work of Lo and Sanwal (44) had indicated a molecular weight of 16K for the dicarboxylate binding protein. Other workers in this laboratory had previously isolated a 56K periplasmic protein by aspartate-Sepharose chromatography using conditions similar to those used here and this protein seemed to be proteolysed during storage at 4°C (T.C.Y. Lo, personal communication). It is possible that the 16K-DBP may be a proteolytic fragment containing the succinate binding domain. The proteolysis may have negated a requirement for porin, and hence binding activity was seen with the 16K species.

The original purification of DBP utilized fairly high ionic strength conditions. SEP would not have been retained by the aspartate-Sepharose under these conditions. The reduced affinity of the SEP for the aspartate-Sepharose affinity matrix suggests two possibilities. First, the reduced affinity may be a reflection of a reduced ability to bind succinate, and proteolysis or the presence of porin could lead to a different elution profile. Second, we may be looking at an entirely different protein that was not observed when the columns were equilibrated with a higher ionic strength buffer.

Based on the data presented in this thesis, a role for SEP in facilitating succinate transport without actually binding succinate can be postulated. The finding that a protein can take part in a transport process without exhibiting substrate binding affinity is not surprising. Porin and the glycerol facilitator are known to play an essential role in various transport processes, without demonstrating any binding affinity for their substrates (1,14). Even though cytoplasmic membrane transport components for histidine and maltose transport are essential

for the transport process, substrate binding to these components has not been demonstrated (16,84).

Succinate is a dianionic compound and, consequently, charge repulsion considerations are particularly important in its transport. Negatively charged groups on the cell surface, in the porin pore, and in the periplasmic space could pose a considerable hindrance to transport. The importance of charge in the porin pore on succinate transport was clearly demonstrated in the different uptake rates in the JF-series porin mutants. It could be that SEP does not contain succinate binding activity but its effect on succinate transport may be indirect, through the blockage of negative charges close to the "mouth" of the porin pore. These charges could interfere with the passage of negatively charged succinate through the pore. Similarly, blocking negatively charged groups within the periplasmic space itself (such as those contained on the membrane derived oligosaccharides (MDO)) could also indirectly facilitate transport of negatively charged compounds such as succinate. This mechanism for increased transport would thus probably be a general mechanism and the enhancement of transport of other negatively charged substrates would also be expected to occur. A greater enhancement may be seen in the case of C_4-dicarboxylic acids because of the relatively high "charge per molecular mass" ratio.

The correlation between SEP binding to whole cells and uptake capability was quite strong. Those cells that were unable to bind SEP were also unable to transport succinate. SEP showed increased binding to mutants previously characterized as either lacking DBP (*cbtB*) or having defective DBP (*cbtA*). This suggests that SEP and DBP may share some common features. Certainly the binding site recognized by SEP

(apparently made up of porin and LPS) could be similar to the binding site recognized by DBP. The possibility exists that SEP may be a precursor of DBP, and that post-translational proteolytic processing is required before dicarboxylate binding activity can be detected. It is also possible that the absence of the 52K protein in BL25 may be an indication of a defect in this processing. The absence of the 52K protein may be a reflection of a general processing defect or, alternately, the 52K protein may itself be part of the processing "machinery". The question of SEP as a DBP precursor could be explored.

A strong possibility exists that the protein identified in some of the previous studies as DBP may in fact be SEP. This is an especially important consideration in some of the localization studies in which the aspartate-Sepharose was equilibrated with low ionic strength buffers (46,58). The cell surface protein, identified as DBP based on its ability to bind to aspartate-Sepharose, could have been SEP. No detailed determination of the affinity of this protein for aspartate-Sepharose were undertaken in these earlier studies. If this cell surface "DBP" is in fact the cell surface SEP, the proposed model of dicarboxylic acid transport in E. coli will have to be revised. In the original model the cell surface DBP was thought to facilitate dicarboxylic acid transport across the outer membrane by providing, in effect, a cell surface substrate binding site for the otherwise nonspecific porin pore (47). A conceptual difficulty with this model is the mechanism by which the high affinity binding site could release its substrate into the pore. The possibility exists that the binding to porin could cause a conformational change, and with correct binding site

orientation, release into the pore. On the other hand if the cell surface population is in reality SEP, the charge blocking mechanism proposed above would account for the uptake stimulating cabability of externally added protein.

A number of studies can be proposed to resolve the question of the possible interrelatedness between SEP and DBP, and in so doing clarify the model and many of the questions raised in this thesis. A panel of monoclonal antibodies raised against this protein could demonstrate similarity between SEP and DBP. The use of recombinant DNA technology could greatly facilitate these studies in that strains overproducing the succinate transport genes could be used in the isolation of the binding protein. Nucleic acid sequence data could be compared to SEP amino acid sequence data to confirm the similarity.

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